

THE RELEASE OF ENZYMES FROM  
DAMAGED MYOCARDIUM

by

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I hereby declare that this thesis has been  
written by me and that the work described  
in it is my own.

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## SUMMARY

This thesis is concerned with the application and development of serum enzyme tests in the diagnosis of myocardial infarction. Two aspects of the problem have been selected for detailed study: firstly, the measurement of the activity of the MB isoenzyme of creatine kinase and, secondly, the release of mitochondrial enzymes in myocardial infarction. In addition, the biochemical properties of the MB isoenzyme have been compared with those of the MM isoenzyme of creatine kinase in an attempt to provide the basis of a simple test for the measurement of serum levels of the MB isoenzyme.

The enzyme tests used in the study were assessed. While, in most cases, existing laboratory methods were found to be suitable, a method of quantitating the MB isoenzyme was developed from a qualitative electrophoretic technique already available, and a new method for the measurement of malate dehydrogenase (MD) activity was developed.

Serum samples were obtained from the following groups: 142 patients with suspected myocardial infarction; 201 patients with disorders, other than myocardial infarction, known to cause elevation of serum total creatine kinase activity; and 132 apparently healthy male blood donors. The last group was used to determine reference ranges for each of the enzymes under investigation.

Serial determination of the serum activity of the MB isoenzyme of creatine kinase, total creatine kinase, aspartate aminotransferase (AST), urea-stable lactate

dehydrogenase (LD), malate dehydrogenase (MD) and alanine aminotransferase (ALT), were made on all patients with suspected myocardial infarction.

The MB isoenzyme of creatine kinase was found to become elevated before any of the other enzymes, abnormal activities being observed as early as three hours after the onset of chest pain. Enzyme activity reached a peak after about 17 hours, significantly earlier than any other enzyme studied, but activity also returned to normal more rapidly than the other enzymes.

The MB isoenzyme of creatine kinase appeared to be a more sensitive index of myocardial infarction, the average peak activity being over 20 times the upper reference value, whereas corresponding figures for total creatine kinase and AST were less than 10 times the upper reference value.

The MB isoenzyme was also found to be more specific for myocardial damage than total creatine kinase. The studies on patients not suspected of having had myocardial infarcts confirmed this greater specificity since, apart from patients after cardiac operations, the raised serum total creatine kinase activity was not accompanied by any elevation of MB isoenzyme activity.

Determination of the activity of glutamate dehydrogenase and the mitochondrial isoenzymes of AST and MD were made on 68 of the patients with unequivocal myocardial infarction. The mitochondrial isoenzymes reached their peak activity later than the corresponding cytoplasmic isoenzymes. Furthermore, although the peak activities

of mitochondrial AST and MD correlated well with one another, they did not correlate well with the corresponding peak activities of the cytoplasmic isoenzymes. Although the mitochondrial isoenzymes were less sensitive indicators of myocardial infarction than the cytoplasmic enzymes, there was some evidence to suggest that the activity of the mitochondrial isoenzymes provided a better index of the severity of the condition, at least insofar as the incidence of heart failure was concerned. There was insufficient evidence to determine whether the pattern of release of mitochondrial isoenzymes differed in important respects from the cytoplasmic isoenzymes. Elevations in serum glutamate dehydrogenase activity were inconsistent and, when present, only small elevations were seen except in patients who had coincident liver damage.

Partially purified preparations of the MM and MB isoenzymes of creatine kinase were prepared from human heart tissue, and the physical and biochemical properties of the two isoenzymes were compared. The MB isoenzyme was more sensitive to denaturation by heat, 8 mol/l urea and extremes of pH than the MM isoenzyme. Under certain conditions, however, the MM isoenzyme was found to be inactivated by p-hydroxymercuribenzoate more rapidly than the MB isoenzyme. The two isoenzymes also had different substrate affinities, the MB isoenzyme having lower  $K_m$  values for MgADP and creatine phosphate. The  $K_m$  values of the two isoenzymes also behaved differently when the pH of the assay medium was altered. In most of the other properties studied

(type of reaction mechanism, behaviour with inhibitors, activation by thiols) the two isoenzymes were similar to one another.

An assessment of some of the currently available methods of assay of the MB isoenzyme of creatine kinase in serum has been carried out. An unsuccessful attempt was made to develop a method based on the differences in behaviour of the two isoenzymes with p-hydroxymercuribenzoate. A simple ion-exchange method, using DEAE Sephadex, was developed and was compared with polyacrylamide and agarose gel electrophoretic techniques. While all three methods were able to give satisfactory results, the ion-exchange method and a commercial electrophoretic method using agarose gel were found to be more precise, accurate and convenient than polyacrylamide gel electrophoresis.

The present studies, in conjunction with those from other laboratories, confirm that measurement of serum activities of the MB isoenzyme of creatine kinase is valuable in the diagnosis of myocardial infarction. It is more doubtful whether the mitochondrial isoenzymes of AST and MD have significant advantages over other enzyme tests. However, knowledge of the factors controlling the release of enzyme from damaged myocardium is still incomplete and further investigations are required.



SECTION 1  
INTRODUCTION

1-1 Multiple forms of enzymes.

Enzymes are named after and identified by their catalytic activities. Different proteins with the same catalytic activity are therefore classified under the same name. In 1895 Emil Fischer advised that tissue and species origin should be reported when describing enzymes, thus implying that there might be tissue and species differences between enzymes. Although many enzymes and co-enzymes have been proved to be very similar (if not identical) despite different biological origins, examples of species differences in enzymes abound in the literature, e.g. aldolase (Warburg and Christian, 1943) and pancreatic amylase (Meyer et al., 1947). Comparative studies of such enzymes are valuable in the investigation of molecular evolution.

In 1948 Warburg suggested that enzymes catalysing similar reactions in different tissues might be organ-specific. This has been found to be true for many enzymes. Alkaline phosphatases, for example, in bone, intestine and liver, differ in electrophoretic mobilities, immunological properties and many other biochemical properties. Other examples include serum and erythrocyte cholinesterases, and prostatic and erythrocyte acid phosphatases.

There are also some enzymes which occur within a species in a number of different forms distributed in different proportions in different tissues. Lactate dehydrogenase is an example in this category. Wieland and Pfleiderer (1957) found that most organs had five protein fractions

with lactate dehydrogenase activity. Later Vesell and Bearn (1961) showed that the electrophoretically fast moving forms predominated in human heart tissue while the slow forms predominated in human liver and skeletal muscle. It has been suggested that such differences may have biological significance (Umbarger, 1961). Thus, Cahn et al. (1962) proposed that the slow forms of lactate dehydrogenase, which are only slightly inhibited by excess lactate, allowed the accumulation of lactate hence favouring the anaerobic respiration which may be found in skeletal muscle. The fast forms, on the other hand, which are more sensitive to inhibition by excess lactate are found predominantly in heart tissue where aerobic respiration is the rule.

The multiple enzyme forms in tissue may be found in the same or different parts of the cell. All five forms of lactate dehydrogenase are present in the cytoplasm. On the other hand, the two different forms of aspartate aminotransferase (Boyd, 1961), malate dehydrogenase (Wieland et al., 1959) and  $\alpha$ -glycerophosphate dehydrogenase are separated from each other, one in the cytoplasm and the other in the mitochondria. Since it is likely that substrate concentrations and metabolic activities in cytoplasm and mitochondria are different, it is hardly surprising that the different isoenzymes present in these two sites might have different kinetic and physical properties.

Markert and Möller (1959) were the first to use the term "isozyme" to describe different proteins with similar catalytic properties. The precise definition of the term

is difficult as many enzymes have broad and overlapping substrate specificities. Different forms of enzymes may arise from genetically independent proteins (e.g. aspartate aminotransferase isoenzymes) or from heteropolymers of two or more polypeptide chains under separate genetic control (e.g. lactate dehydrogenase). Apart from these genetic causes, multiple enzyme forms may arise from:

1. proteins being conjugated to different groups (e.g. phosphorylases a and b);
2. proteins being derived from one parent polypeptide chain (e.g. family of chymotrypsins from chymotrypsinogen);
3. polymerisation of a single subunit (e.g. glutamate dehydrogenase);
4. conformational changes in protein molecules (all allosteric modifications of enzymes);
5. artifacts arising from the preparation and separation of the enzyme.

In 1964, the Standing Committee on enzymes of the International Union of Biochemistry stated that the term, isoenzyme or isozyme, should only be applied to different enzymatic forms within a species arising from genetically determined differences in the primary structure.

The study of isoenzymes within and among tissues has an important application in clinical medicine. It has been found that isoenzyme patterns not only vary with species and tissues, but also with age and in pathological conditions. Schapira et al. (1968) pointed out that there was a general

tendency for isoenzymes to revert to their foetal pattern in cancerous and atrophic tissues. This might be taken as an expression of tissue de-differentiation at molecular level. It is possible that more can be discovered about both the progress and the molecular aetiology of some diseases by studying the isoenzyme patterns of the affected tissues. Moreover, during tissue necrosis enzymes are released into the circulation and become detectable in the body fluids.

By measuring the activity of the released enzymes more information about the nature of the disease can be obtained. The value of such enzyme determinations may be greatly increased by isoenzyme studies which help to demonstrate the tissue of origin of the enzyme so that the site as well as the extent of the disease can be more accurately assessed. The work described in this thesis is concerned with these clinical applications of isoenzyme measurements.

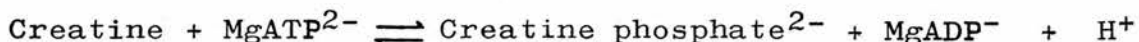
The release of the isoenzymes of creatine kinase, aspartate aminotransferase and malate dehydrogenase from damaged human myocardium has been studied with a view to determining their clinical value in their determination in serum.

#### 1-2 Isoenzymes of creatine kinase.

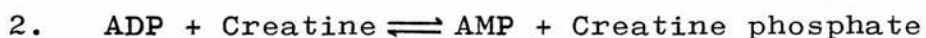
(ATP: creatine phosphotransferase EC 2.7.3.2.)

1-2-1 Discovery of creatine kinase and its isoenzymes.

Creatine kinase catalyses the reversible phosphorylation of creatine by adenosine triphosphate in the presence of  $Mg^{2+}$  ion.

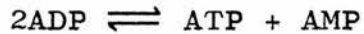


The discovery of the enzyme came as a result of the interest in inorganic phosphates in muscle. In 1927 Fiske and Subbarow found that much of the inorganic phosphate in muscle filtrates arose from acid hydrolysis of an organic phosphate. They considered that their organic compound contained equimolar amounts of creatine and phosphate. Lohmann in 1928 found that a considerable part of the organic phosphate was linked to adenylic acid and was relatively stable to acid. A few years later Lohmann (1934) was able to demonstrate the presence of an enzyme catalysing the transfer of a phosphate group from creatine phosphate to adenylic acid forming "adenyl pyrophosphate". The reverse reaction, the transfer of phosphate from ATP to creatine, was soon shown by Needham and Van Heyningen (1935). Lehmann (1935, 1936) considered the reaction to consist of two stages



However, Banga in 1943 reported that the partially purified muscle extract was unable to catalyse reaction 2 after the 57% ethanol precipitation stage. She concluded that the two reactions were catalysed by different enzymes. The situation was clarified by Chappell and Perry (1953) who showed that muscle extract in the absence of adenylate kinase was unable

to catalyse reaction 2. Adenylate kinase catalyses the reaction



Reaction 2 was, therefore, the combined effect of the creatine and adenylate kinases.

Although most of the pioneering work on creatine kinase was done with muscle extracts, the enzyme is also present in high concentration in the brain. Narayanaswami (1952) and later Wood (1963) showed that the brain enzyme had different electrophoretic mobilities and substrate affinities from the muscle enzyme. Electrophoretic studies in vertebrates (Deul and van Bremen 1964, Rosalki, 1965) showed that cytoplasmic creatine kinase occurred in three distinct forms - one with high anodal mobility found mainly in brain tissue, one with low anodal mobility present in muscle and a third one with intermediate mobility mainly present as a minor component in heart extracts. Dawson et al. (1965) confirmed that the form with intermediate mobility was a hybrid of the electrophoretically fast and slow isoenzymes. They subjected a mixture of purified fast and slow forms to dissociation with 6.5mol/l guanidine hydrochloride. After reactivation by dialysis, electrophoresis showed the presence of the hybrid between the two parent forms. The three band pattern suggested that the enzyme existed as a dimer. According to their main tissue of origin the three forms were named the muscle type (MM), the brain type (BB) and the hybrid (MB).

All these isoenzymes have been shown to occur in the cytoplasm. However, other forms of creatine kinase with

properties different from the soluble isoenzymes have also been found. Jacobs et al. (1964) demonstrated creatine kinase activity in purified mitochondria from skeletal muscle, cardiac muscle and brain. Ottaway (1967) purified creatine kinase from the myofibrillar fraction of ox heart muscle. Keto and Doherty (1968) crystallised creatine kinase from a non-mitochondrial particulate fraction of a pig heart extract. The sarcoplasmic reticulum of rabbit skeletal muscle has also been reported to contain creatine kinase (Baskin and Deamer, 1970) representing 1% of the total tissue enzyme, but no properties have been found to distinguish it from the MM cytoplasmic isoenzyme. To date relatively little is known about these particulate enzymes. The present work is only concerned with the cytoplasmic forms.

#### 1-2-2 Comparative aspects of the cytoplasmic isoenzymes.

Like lactate dehydrogenase isoenzymes, the isoenzyme patterns of creatine kinase change during both ontogeny and phylogeny.

Of the thirteen species studied by Eppenberger et al. (1970), the isoenzyme pattern in the myocardium was found to be the most variable of all tissues. In man, the myocardium contained a major band of the MM isoenzyme and a weak band of the MB isoenzyme. In rabbit, only the MM isoenzyme was found. In rat all three isoenzymes were present, with the MM isoenzyme as the major band. In chicken heart tissue the BB isoenzyme predominated with a weak band of the MB



isoenzyme. Some avian species had two brain-type isoenzymes which, upon hybridisation with the muscle enzyme, gave two hybrid bands. By hybridisation and optical rotary dispersion studies, it has been shown that the brain isoenzyme is structurally less organised than the muscle enzyme and can therefore give rise to slightly different molecular forms.

Hybridisation of the BB and MM isoenzymes is not confined within a species. Interspecies MB isoenzyme hybrids have been formed from mixtures of cat, human and chicken tissue extracts (Dawson et al., 1967). Indeed, hybridisation between the BB isoenzyme of rabbit and the dimer arginine kinase of sea cucumber has been demonstrated (Watts et al., 1972). These examples show the close structural phylogenetic relationship of phosphagen kinases among species. This is made more evident by protein structure studies.

The MM isoenzymes of different species are more similar in their primary, secondary and tertiary structures than are the MM and BB isoenzymes within a single species (Gosselin-Rey and Gerday, 1970). In ox, rabbit and chicken the BB isoenzymes are all known to contain significantly fewer basic amino acids, and more cysteine and aromatic amino acids than the MM isoenzymes (Noltmann et al., 1962). The presence of fewer basic amino acids explains the greater anodal electrophoretic mobility of the BB form at pH values above neutrality. The hybrid MB isoenzyme has an amino acid composition intermediate between its parent forms (Eppenberger et al., 1967). Antisera raised against the MM isoenzyme will not cross-react with the BB isoenzyme and vice versa, but will

react with the same isoenzymes from other species (Bulcke and Sherwin, 1969). Sedimentation and other studies have confirmed that the isoenzymes consist of two freely dissociable subunits, each consisting of a single polypeptide chain having no disulphide bridges (Yue et al., 1967). The subunits are cigar-shaped and lie side by side. It is believed that all three isoenzymes have this shape, which is strongly retained among phosphagen kinases throughout evolution.

The changes of isoenzyme pattern with ontogeny have been studied by Eppenberger et al. (1964). They demonstrated in rat and chicken embryos that the brain-type isoenzyme (BB) appeared first in heart, skeletal muscle and brain tissues. In brain the BB isoenzyme remained the only type of creatine kinase throughout development. In heart and skeletal muscle the BB isoenzyme was gradually replaced by the MB isoenzyme which was in turn replaced by the MM type. These transitions correlated with morphological and physiological changes. Eppenberger et al. (1964) divided this development into four stages:

1. The myoblast stage when the BB was the only isoenzyme present. The MB isoenzyme began to appear when myocells were formed.
2. The myotube stage when the cells showed fibrillation and cross-striation. The MB isoenzyme was the predominant creatine kinase at this stage.
3. The myofibril stage, characterised by a rapid rise of actomyosin and creatine kinase activity. The BB isoenzyme then disappeared with the myotubes

and the MM isoenzyme became more and more pre-dominant.

4. The final stage was marked by further increase of creatine kinase, actomyosin and creatine phosphate. By this stage the MB isoenzyme had been completely replaced by the MM isoenzyme. In mature myocardium, however, the MB isoenzyme still remained in significant concentration. This may be attributed to the morphological differences between skeletal muscle and myocardium. Although the myocardium contains contractile proteins similar to those of adult skeletal muscle, many of its structural properties resemble more those of the embryonal muscle of the myotube stage when both the MM and MB isoenzymes are present.

Reversion to the foetal-stage isoenzyme pattern has also been observed in various forms of muscular dystrophy. The exact mechanism of this reversion is still not clear.

#### 1-2-3 Physiology of creatine kinase isoenzymes in human myocardium.

Energy liberation, conservation and utilisation are three important phases in the metabolism of normal myocardium. The cardiac muscle takes up from blood energy-containing compounds which include fatty acids, pyruvate, lactate, acetoacetate and glucose. The energy liberated from the catabolism of these substances is largely stored

in the form of "high-energy" phosphate compounds whose breakdown provides energy for the contractile mechanism of the muscle cell for the performance of mechanical work. The heart is well adapted for continuous aerobic work since it is 3 - 4 times more vascular and has 10 - 20 times more blood-flow per unit weight of tissue than has skeletal muscle (Olson and Schwartz, 1951). It is rich in myoglobin and has a very high concentration of mitochondria.

Myocardial creatine kinase is mainly concerned with energy storage. The enzyme maintains a transphosphorylation equilibrium between ATP and creatine phosphate which together constitute 90% of the "energy-rich" phosphate bonds in cardiac muscle (Furchgott and Lee, 1961). At pH 7.0 the free energy made available by hydrolysis of creatine phosphate is about 6300 J/mol greater than that of ATP, thus the equilibrium favours the formation of ATP. ATP is the major energy source for muscle contraction while creatine phosphate acts as an energy reserve. Any ATP used up is rapidly replenished by creatine phosphate through the action of creatine kinase. Interference with oxidative metabolism has been found to result in a decrease in creatine phosphate concentration, ATP levels being more slowly affected (Furchgott and Lee, 1961). The free energy of hydrolysis of creatine phosphate cannot be made directly available to the contractile element, actomyosin. Nevertheless, it has been found that, in the absence of any added nucleotide and in the presence of creatine phosphate, the unpolymerised globular form of actin (G-actin), when complexed with ADP, can function as

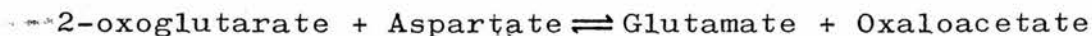
substrate for creatine kinase and form a single molecular complex with the enzyme (Yagi and Noda, 1960). In vivo, ADP bound to actin may, therefore, be directly phosphorylated to ATP during each contractile cycle.

The physiological significance of the presence of different creatine kinase isoenzymes in the myocardium is still not clear. It is not certain if the cytoplasmic MM and MB isoenzymes serve different functions, or if the MB isoenzyme is no more than an embryonic vestige. The latter interpretation seems more likely as the MB isoenzyme is absent in the myocardium of many other species.

Attempts have been made to assign a different function to the mitochondrial creatine kinase. This isoenzyme has been found to react only with extra-mitochondrial adenine nucleotides. Since it is capable of regenerating ADP from extra-mitochondrial ATP in the presence of creatine, it has been suggested that it might help to regulate mitochondrial respiration in response to the requirements of muscle contraction (Bessman and Fonyo, 1966).

1-3 The isoenzymes of aspartate aminotransferase (L-aspartate: 2-oxoglutarate aminotransferase EC 2.6.1.1).

Aspartate aminotransferase is the most abundant of the numerous aminotransferases in human tissues. Occurring in most major human tissues, especially in heart, liver and skeletal muscle, it catalyses the reaction



in the presence of pyridoxal phosphate as co-enzyme. The enzyme occupies a central role in both anabolism and catabolism of amino acids and serves as a link between amino acid and carbohydrate metabolism. At physiological pH the reaction equilibrium favours the formations of glutamate and oxaloacetate.

The enzyme has at least two fractions separable by electrophoresis, as first reported by Green et al. (1945). The cationic fraction has been shown to originate from the mitochondria and the anodic fraction from the cell cytosol (Hird and Rowsell, 1950; Boyd, 1961). The two isoenzymes differ greatly in many biochemical properties:

1. They have different amino acid compositions, although their molecular weights are similar (Martinez-Carrion et al., 1967).
2. Antisera raised against one isoenzyme do not cross-react with the other (Nisselbaum and Bodansky, 1964).
3. They have different kinetic parameters. For example, the mitochondrial isoenzyme has a lower Michaelis constant ( $K_m$ ) for aspartate, whereas the cytoplasmic form has a lower  $K_m$  for 2-oxoglutarate.
4. The mitochondrial isoenzyme is stable in acid pH 5-7 while the cytoplasmic isoenzyme is not.
5. The cytoplasmic isoenzyme is appreciably more stable to heat (Wada and Morino, 1964).

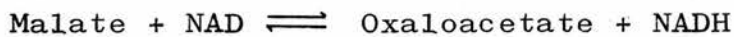
There is evidence which suggests that the two isoenzymes are not homogeneous. Three separate bands in the cytoplasmic isoenzyme and two in the mitochondrial isoenzyme have been



reported in human sera using starch gel electrophoresis (Block et al., 1964). It is not certain whether the occurrence of two isoenzymes and their sub-forms has any biological significance.

1-4 Isoenzymes of malate dehydrogenase (L-malate: NAD oxidoreductase EC 1.1.1.37).

Malate dehydrogenase catalyses the reaction



The enzyme catalyses one of the stages in the aerobic oxidation of carbohydrates via the tricarboxylic acid cycle. At physiological pH the equilibrium favours the formation of malate. Like aspartate aminotransferase it is heterogeneous, occurring in cytoplasmic and mitochondrial forms, each of which is further separable by electrophoresis into 5 or 6 sub-bands of identical catalytic activity (Thorne et al., 1963). The exact nature of these sub-forms is unknown.

At pH 6.2 the soluble (cytoplasmic) isoenzyme migrates towards the anode and the mitochondrial isoenzyme towards the cathode during electrophoresis on starch gel. The isoenzymes have different substrate affinities and turnover rates. Both forms are strongly inhibited by excess substrate, the mitochondrial form by oxaloacetate and the cytoplasmic form by malate. It is thought that these characteristics may prevent the reduction of oxaloacetate in mitochondria and the oxidation of malate in the cytoplasm.

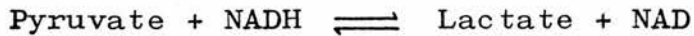
Based on this, different roles have been assigned to the two forms of enzyme (Kaplan, 1961). The cytoplasmic isoenzyme couples NADH oxidation with the reduction of oxaloacetate to malate. The malate thereby produced enters the mitochondria, where it is oxidised by the mitochondrial isoenzyme in the presence of the mitochondrial bound NAD. The oxaloacetate regenerated may then be released into the cytoplasm enabling the cycle to continue. In this way the two isoenzymes help regulate the NAD/NADH ratio within the mitochondria.

1-5 Isoenzymes in the diagnosis of myocardial infarction.

1-5-1 Biochemical changes in myocardial infarction.

Ischaemia produced by coronary occlusion disturbs myocardial metabolism by interfering with energy production. Most of our biochemical knowledge about myocardial ischaemia comes from animal experiments. It has been shown that following complete ligation of the coronary arteries there is a rapid decline in the ATP, creatine phosphate and glycogen content of myocardial cells. Simultaneously there is a rise in lactate and  $\alpha$ -glycerophosphate concentration indicating a shift to anaerobic metabolism. As the oxygen tension in the myocardium falls, the oxidation-reduction systems shift towards a more reduced state. The NADH/NAD system is one of the systems involved. As a result, the concentration of NADH rises and the lactate/pyruvate system, having a redox potential slightly higher than that of NADH/NAD, is the next to be reduced in the presence of lactate dehydrogenase.





In the presence of anoxia this allows other metabolic systems needing NAD to continue to function. If the anoxia is not rectified, the whole energy production system will soon be severely impaired. Consequently, the muscle cell can no longer maintain the ionic gradients across the cell membrane. Cell permeability rises, and various intracellular substances including enzymes leak from the cell. This may happen before the metabolic processes are irreversibly damaged. The contractile fibril, actomyosin, fails to dissociate to reverse muscular contraction. Abnormal cross-linkages are formed between different filaments. At this stage cell necrosis sets in.

A series of glycolytic and oxidative enzymes has been studied in myocardial infarction (Gudbjarnason et al., 1967). It was found that following infarction there was a rapid decline in mitochondrial enzyme activity in the infarcted tissue, with the lowest level in the centre of the infarct. The glycolytic enzymes also decreased significantly in activity, for example, aldolase, lactate dehydrogenase and glyceraldehyde phosphate dehydrogenase decreased to 14%, 21% and 37% of their original activity respectively. In contrast, the activities of the hexose-monophosphate-shunt enzymes rose in experimental myocardial infarction. Glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase rose 34-fold and 10-fold respectively in the affected heart tissue. This enhanced hexose-monophosphate shunt activity implies the adaptive synthesis of ribose and ribonucleic acids in the presence of injury.

1-5-2      Enzymes in the diagnosis of myocardial infarction.

With the common occurrence of this disease, its diagnosis and prognosis have become a major subject of interest to cardiologists. The current diagnostic methods based on clinical assessment and the electrocardiogram are not altogether satisfactory for the following reasons (Coodley, 1970):

1. 25% to 30% of myocardial infarcts are not diagnosed ante mortem.
2. Clinical diagnosis and angiographic studies do not correlate in 25-30% of patients.
3. Electrocardiographic findings (ECG) may not be helpful if:
  - a. Prior left bundle branch block is present.
  - b. Old changes exist that may obscure the current ECG pattern.
  - c. The infarct is intramural, since intramural infarcts may not change the ECG pattern.
  - d. The infarct is diaphragmatic, since diaphragmatic infarcts are often missed in the ECG.

In 1954 LaDue, Wroblewski and Karmen were the first to report a rise of aspartate aminotransferase activity in the sera of patients after myocardial infarction; the amount and duration of the elevation roughly correlated with the size of infarct. Since then, lactate dehydrogenase, creatine kinase, malate dehydrogenase and a variety of other enzymes have all been reported to be present in increased amounts in serum after infarction, and have been used as routine diagnostic tests for myocardial infarction. However, these tests are not as

satisfactory as originally hoped.

The inherent problem is the lack of tissue specificity of the enzymes, since they are also released in a number of other disease states. For example, aspartate aminotransferase, though rich in heart muscle, occurs in all tissues of the body. Besides myocardial infarction, it is released into the circulation in hepato-biliary diseases, severe tachycardia, pulmonary embolism, intravascular haemolysis, muscle injury, acute pancreatitis and extensive surgical operations. The same is true for lactate dehydrogenase - its activity in serum is raised in hepatic, malignant, haematological, muscular and renal diseases as well as myocardial infarction. Moreover, normal results of lactate dehydrogenase have been reported in a number of proven cases of myocardial infarction (Freeman et al., 1959). Even creatine kinase, which was first thought to be more specific to myocardial infarction than any other enzyme, has also been found raised in the serum in muscular dystrophy, neurological and psychiatric disorders, pulmonary and endocrine diseases as well as in a number of physiological states. This lack of specificity means that false positive results are frequent if these enzymes are used uncritically in diagnosing myocardial infarction.

1-5-3

#### Use of isoenzymes.

Various attempts have been made to improve the specificity of serum enzyme tests. The application of lactate

dehydrogenase isoenzyme patterns in tissue to diagnostic medicine has had considerable success. The distribution of the five isoenzymes was studied in several human tissues and listed as follows:

Lactate Dehydrogenase Activity Obtained by  
Starch Block Electrophoresis

% Total Activity in Each Peak

Tissue	LD1	LD2	LD3	LD4	LD5
Heart	73	24	3	0	0
Liver	4	8	27	24	37
Skeletal muscle	0	0	5	17	78
Kidney	42	45	11	2	0
Red blood cells	43	44	12	1	0
White blood cells	12	49	33	6	0

(After Bodansky, 1967)

From the table it is clear that the isoenzyme pattern of lactate dehydrogenase differs in human tissues. Wroblewski and Gregory (1961) suggested that the measurement of individual isoenzyme components might serve to differentiate various disease processes and that, by measuring differentially the fast-moving lactate dehydrogenase isoenzymes, false positive diagnoses of myocardial infarction would be very much reduced. However, such studies do not offer complete tissue specificity. The isoenzymes are also released in viral myocarditis, active rheumatic carditis and a number of non-cardiac conditions including megaloblastic and haemolytic anaemia and progressive muscular dystrophy.

The study of lactate dehydrogenase isoenzymes has given impetus to the investigation of other isoenzymes in the hope of further improving the tissue specificity and sensitivity of the tests. Several studies of the mitochondrial isoenzyme of aspartate aminotransferase have been reported. Preliminary studies have shown that the isoenzyme is raised in the sera of patients with myocardial infarction (Fleisher et al., 1960; Boyde and Latner, 1962). Boyde (1968a) found that the raised serum activities of the mitochondrial isoenzyme returned to normal at a slower rate than total enzyme activity. In eight cases the isoenzyme activity was found raised when the total serum aspartate aminotransferase activity was still normal and hence the author concluded that the mitochondrial isoenzyme might be a more sensitive indicator of myocardial infarction than total enzyme activity. However, reports by other investigators (Fleisher and Wakim, 1963; Massarat and Lang, 1965) did not agree with the findings of Boyde. Much of the evidence gathered so far is conflicting.

The application of creatine kinase to diagnostic medicine came almost a decade later than lactate dehydrogenase and aspartate aminotransferase. When it became clear that total creatine kinase activity was not as specific to myocardial infarction as first expected, mainly due to its release from skeletal muscle in a number of physiological and pathological conditions, efforts were directed towards the use of isoenzymes. The MM isoenzymes in heart and skeletal muscle have been compared, and found to be very similar in all the biochemical properties studied (Smith, 1970), and hence of no

value in the selective diagnosis of myocardial infarction. On the other hand, it was shown that the MB isoenzyme was released into the circulation in myocardial infarction (Van der Veen and Willebrands, 1966; Menache et al., 1968). Biochemical data and clinical experience about the potential value of the MB isoenzyme in diagnosis were lacking when the present project started in 1972.

1-6                    Objectives of the present study.

As discussed in the previous subsection, the data about the clinical use of mitochondrial isoenzymes and the MB isoenzyme of creatine kinase are few and confusing. It has been the overall objective of the present investigation to study the release of these isoenzymes following myocardial infarction and to see if this knowledge would form the basis of an improved clinical test for the diagnosis of myocardial infarction.

It was hoped that the study of the MB isoenzyme would improve the specificity and sensitivity of creatine kinase measurement in clinical laboratories. Also by comparing the release of mitochondrial enzymes (as represented by the isoenzymes of aspartate aminotransferase and malate dehydrogenase, and glutamate dehydrogenase) with those of cytoplasmic origin (lactate dehydrogenase, isoenzymes of aspartate aminotransferase and malate dehydrogenase) it was hoped that more insight might be gained into the potential value of mitochondrial enzymes in indicating tissue damage in myocardial infarction.

It has also been the aim of the present work to study the biochemical properties of the MB isoenzyme of human creatine kinase and to compare them with the more abundant MM isoenzyme. The MB isoenzyme has been neglected in many biochemical studies of the creatine kinase isoenzymes. It is usually regarded as having properties intermediate between its parent forms. The present investigation may both help to fill part of this gap and form a basis for a differential assay of the MB isoenzyme.

The study is divided into three parts. The first deals with the methods of measuring several enzymes in serum. The second part is concerned with studies of the release of the enzymes into serum in patients with suspected myocardial infarction. The third part is a comparative study of the cytoplasmic isoenzymes of creatine kinase, their enzymatic properties and their differential determination.



## SECTION 2

ENZYME ACTIVITY ASSAY IN SERUM AND TISSUE EXTRACTS



In this section the principles and difficulties of methods of measuring creatine kinase, aspartate aminotransferase, malate dehydrogenase and other enzymes investigated in the present study are discussed.

## 2-1      Nature of enzyme activity determinations.

The activity of an enzyme is determined by the catalytic state and by the concentration of the enzyme. By using the same amount of enzyme and varying the experimental conditions, enzyme activity measurements provide useful information about the reaction mechanism and the enzyme active site(s). On the other hand, by keeping the assay conditions constant, enzyme activity measures the amount of enzyme in the reaction mixture. With few exceptions, activity determinations are the only means of detecting enzymes, and hence become the main concern of the clinical biochemist who is, in general, primarily only interested in enzyme concentration. Therefore, enzyme activity measurements are important for both pure and applied studies of enzymes.

### 2-1-1      Theoretical considerations.

Enzyme activity is expressed as the velocity of the reaction it catalyses. The conventional method of following the reaction velocity is to measure the disappearance of substrate, or the appearance of products, as a function of time. The velocity at any particular time is obtained by

drawing a tangent to the concentration-time curve (Fig. 2-1a). The initial velocity (i.e. velocity at the beginning of the reaction) should be used for enzyme activity measurement in order to avoid any complications that may arise during the reaction, such as the effects of the reverse reaction, build-up of inhibitory products, exhaustion of substrates and the possible inactivation of enzymes at the pH and temperature of the reaction.

In most cases the initial velocity of an enzyme reaction is directly proportional to the amount of enzyme present. This relationship forms the basis of nearly all methods of determination of enzyme concentration. However, Dixon and Webb (1964) have listed a number of practical situations when this rule does not hold:-

1. The capacity of the assay is exceeded.
2. Co-factors needed for the reaction in the assay solution are exhausted owing to the presence of an excessive amount of enzyme.
3. The presence of toxic impurities in the reagents.
4. The presence of a reversible inhibitor or activator in the enzyme preparation.

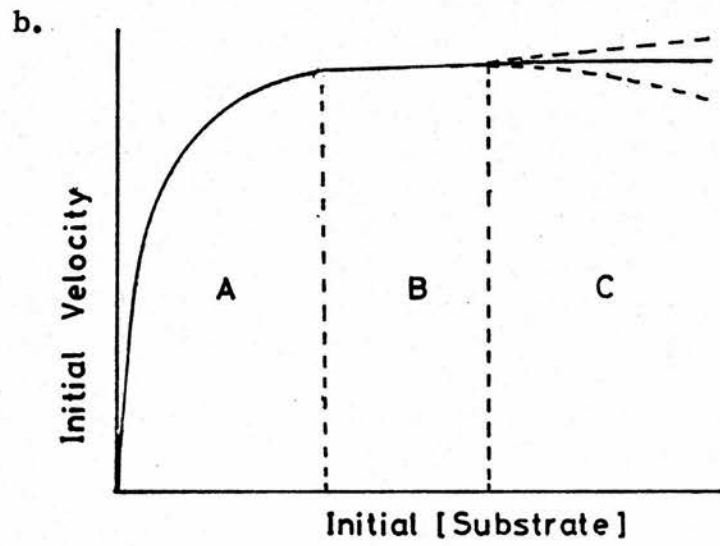
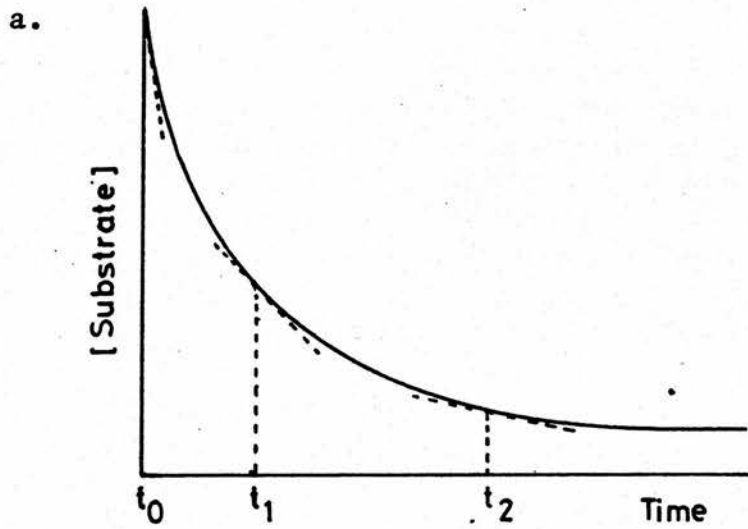
These possibilities should, therefore, be eliminated first before the initial velocity is taken as a measure of enzyme concentration.

In a chemical reaction the relation between the reaction rate and the substrate concentration is expressed by the equation

$$v_o = kS^n$$

Figure 2-1

The Velocity of Reactions Catalysed by Enzymes



where  $v_0$  = initial velocity;

$S$  = substrate concentration;

$k$  = a constant;

$n$  = order of the reaction with respect  
to  $S$ .

In an enzyme reaction,  $v_0$  is first order with respect to  $S$  at low concentrations of  $S$ , but becomes zero order when  $S$  increases and saturates all the enzyme molecules. At still high concentrations of  $S$ , inhibition (or, less frequently, activation) may occur (Fig. 2-1b). From an analytical point of view it is always advisable to measure enzyme activity in the presence of an excess of substrate (Zone B Fig. 2-1b), firstly to ensure that there is no significant fall of substrate concentration during the time of assay, and secondly to minimise instrumental error in delivering substrate, as the reaction velocity is not sensitive to minor variations of substrate concentration in this zone. However, if Zone B is narrow, the fall in substrate concentration may cause a change from zero order to first order reaction conditions within the period of measurement. The activity so obtained is then no longer a true estimation of enzyme concentration.

## 2-1-2

### Methods.

In general, two classes of methods are used to measure enzyme activity. In sampling methods, portions of reaction mixture are taken out at intervals during incubation and

analysed separately to give a number of points representing the progress of the reaction. If the reaction is assumed to be linear, only two points are needed. In continuous methods, the reaction is followed continuously in the reaction vessel. Of the two methods the continuous method is usually to be preferred whenever practicable as it gives a more reliable and accurate estimation of the initial velocity.

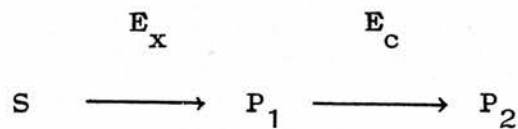
The principal measuring techniques involved are:-

1. Spectrophotometry. Many reaction substrates and products absorb light in the UV and visible region. Using a suitable spectrophotometer, the absorbance can be monitored and the rate of appearance or disappearance of the light-absorbing material can be calculated quantitatively.
2. Fluorimetry. The fluorescence of substrate or product or their derivatives is measured. It is in most cases many times more sensitive than the spectrophotometric technique.
3. pH titration for reactions involving  $H^+$  production or consumption. The change of pH as the reaction proceeds may be monitored or alternatively the pH may be kept constant by continuous titration.
4. Miscellaneous. In addition to the above, there are other more specialised methods meeting the special requirements of certain types of enzyme reactions. Examples are manometric methods for gaseous reaction components, polarimetric methods for changes of optical rotation, and platinum electrode methods for measuring redox potential changes

in oxido-reduction reactions.

At present, the spectrophotometric techniques are most commonly used, mainly as a result of the work of Warburg and Christian (1936) on the coenzymes NAD and NADP. Many of the reactions follow the oxidation/reduction of NADH/NAD by monitoring absorbance at 340nm where NADH and NADPH have an absorption maximum not shown by the corresponding oxidised form of the nucleotide. Not only can oxido-reduction reactions be followed at 340nm, but also other reactions which are able to be coupled to reactions involving NADH or NADPH, e.g. creatine kinase and aspartate aminotransferase.

The general sequence of the coupled reaction may be written as



S = substrate for  $E_x$

$E_x$  = the enzyme whose activity is to be measured

$P_1$  = product of  $E_x$

$E_c$  = coupling enzyme which acts on  $P_1$

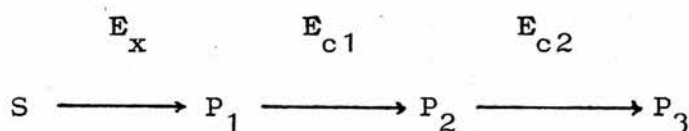
$P_2$  = product of  $E_c$  whose formation involves the  
NADH/NAD or NADPH/NADP systems

$v_x$  = reaction rate from S to  $P_1$

$v_c$  = reaction rate from  $P_1$  to  $P_2$

When the reaction catalysed by  $E_x$  is started,  $P_1$  begins to form. As  $P_1$  accumulates,  $v_c$  begins to rise in the presence of  $E_c$  until a steady state,  $v_c = v_x$ , is reached. As the measured activity is  $v_c$ , it is important that the steady state be reached as soon as possible since the shorter this

lag phase, the more reliable is the estimation of the initial activity of  $E_x$ . The velocity ( $v_c$ ) of the secondary reaction is maximal when  $E_c$  has a low Michaelis constant for the substrate ( $K_{mc}$ ), so that large amounts of  $P_1$  are not required to "drive" the reaction, and when  $E_c$  is present in high concentration, i.e. when  $E_c / K_{mc}$  is large. Therefore, the larger ratio,  $\frac{E_c}{K_{mc}} / \frac{E_x}{K_{mx}}$ , the faster is  $v_c$  increasing from zero to the value of  $v_x$ . To achieve this, an amount of  $E_c$  many times in excess of  $E_x$  is needed and a coupling enzyme with a low  $K_m$  should be preferred if possible. The same principle applies to systems with more than one coupled reaction:



If care is taken to ensure that the ratios  $E/K_m$  of  $E_{c1}$  and  $E_{c2}$  are many times larger than that of  $E_x$ , the lag phase can be short and a linear reaction course can be obtained. However, in using highly active purified coupling enzymes, there is the inherent danger of any essential cofactors in the assay system being exhausted by these enzymes, and of introducing undesirable materials as impurities (such as substrates or enzymes) giving rise to side reactions. So, in selecting a coupled-system assay, one has to balance the advantages against these disadvantages.

## 2-1-3

### Sources of error.

Recent advances in techniques of enzyme measurements

have improved the precision, accuracy and sensitivity of enzyme assay methods, but the variables affecting enzyme catalysis are still not fully under control. While it is relatively easy to control substrate concentration, pH, temperature, ionic strength and nature of the buffer, other variables are more difficult to control. These include:-

1. denaturation of enzyme on the surfaces of the reaction vessel and delivering pipettes,
2. inactivation around liquid-air interfaces,
3. presence of traces of toxic or activating substances,
4. presence of interfering enzymes in the reaction mixture,
5. possible dissociation of enzyme into inactive proteins on dilution,
6. changes of enzyme properties due to ageing,
7. conditions of storing enzyme solutions,
8. human and instrumental errors involved in the assay.

For these reasons it is very difficult to prepare a satisfactory reference enzyme material to standardise techniques of enzyme measurements. Instead, efforts have been directed towards obtaining reliable reference methods of analysis; this should eliminate many intra-laboratory and some inter-laboratory sources of errors. When enzyme activity is shared by a number of proteins, it is unlikely that the assay conditions are optimal for all the enzyme proteins concerned. The activity measurement may then present a biased picture of the various contributions of the isoenzymes unless the isoenzymes are differentially measured at their own



individual optimal conditions.

2-1-4

Units of enzyme activity.

Differences in the units used to express the results of determinations of enzyme activity have caused much confusion. The units of activity were often arbitrarily defined by the originators of the assay methods and even by different workers using the same procedures. The Report of the Commission on Enzymes (1961) of the International Union of Biochemistry attempted to clarify the situation by recommending a standard enzyme unit, the international unit (IU). One unit of activity of any enzyme is defined as "that amount which will catalyse the transformation of 1 micromole of substrate per minute, or where more than 1 bond of each substrate molecule is attacked, 1 micro-equivalent of the group concerned per minute, under defined conditions". Since the unit does not define the temperature of the reaction nor the variables of the reaction mixture, the degree of uniformity brought about by the proposal has been limited. It is very likely that different values in international units will be obtained when the same amount of enzyme is measured by two different procedures even if temperature differences are corrected for. This is due to the fact that the two procedures are probably measuring different facets of enzyme activity.

Recently, another enzyme unit has been proposed as the basic unit of enzyme activity. This is the Katal, which is

mol/sec of substrate transformed. Throughout the present work, however, all enzyme activities are express in IU/litre at 37°C, unless stated otherwise.

## 2-2 Enzyme measurements in human serum.

A substantial part of the present study is concerned with enzyme assays in serum. Serum is usually preferred to plasma in most enzyme assays. One advantage is that there is no delayed clot formation in serum such as often occurs in stored plasma samples. Also, no anticoagulants, which may be toxic to one or more enzymes, are present in serum. However, it should be noted that platelet enzymes are released into serum as a result of coagulation. Because of the nature and composition of serum, special factors additional to those mentioned in the last sub-section have to be considered in the design of experiments for measuring enzyme activity in this medium:

1. The buffering properties of serum. Serum is a strong buffer acting over a fairly wide pH range. It contains bicarbonate, phosphate, amphoteric amino acids and proteinate ions. If added in appreciable quantities, it can alter the pH of the assay mixture. As the buffer properties of serum change greatly with pathological states, no accurate estimation of its effect on the pH of the reaction mixture can be made. The choice of a buffer of appropriate strength, therefore, becomes a matter of great importance.

2. Presence of enzyme substrates in serum. Serum contains an appreciable quantity of metabolites such as lactate, pyruvate and other ketoacids, etc. They are the substrates of a number of enzymes, and their presence in the reaction mixture may give a high, but variable blank activity. To eliminate this effect, the serum sample should be pre-incubated with the reaction mixture for a period long enough to exhaust all the endogenous substrates before the enzyme reaction proper is started with an exogenous substrate. It should also be ensured that no exogenous co-enzymes or substrates in the reaction mixture are decreased to a sub-optimal level as a result of taking part in the endogenous substrate reaction, e.g. the consumption of NADH by lactate dehydrogenase and pyruvate in serum in the assay of alanine aminotransferase.

3. Presence of specific and non-specific enzyme inhibitors. The inhibitors in serum may include heavy metal ions such as  $\text{Cu}^{2+}$  and  $\text{Hg}^{2+}$ , organic acids, inorganic anions, and protein macromolecules (antienzymes). Their concentrations may vary between individuals and with differing physiological and pathological states. The inhibitors may occur naturally or be of exogenous origin, e.g. drugs.

4. Presence of proteinases in serum. These proteolytic enzymes may hydrolyse enzyme proteins in serum. This constitutes one of the possible causes of the fall of serum enzyme activity on storage. To prevent this, activity should be measured as soon as possible but if storage is necessary, the serum samples should be refrigerated or

frozen.

5. Presence of bacteria in serum. The bacteria may be present endogenously in serum in certain pathological states or, more commonly, they may be introduced into the samples during collection and storage. The bacteria have their own patterns of enzyme activity and they may excrete or secrete toxins. Besides synthesising and secreting enzymes which may catalyse reactions similar to serum enzymes, bacteria may also cause breakdown of serum enzymes and consumption of substrates and co-factors. The effects of bacterial contamination are such that any enzyme activity data obtained from contaminated samples are meaningless.

6. Haemolysis and lysis of white blood cells and platelets. Since both erythrocytes and leucocytes are richly endowed with enzymes, their lysis will greatly distort the enzyme concentration and distribution pattern in serum. The lysis of these blood cells may occur in vivo in some pathological states or, more commonly, in vitro due to mis-handling of the blood samples.

7. Venous stasis occurring during sample collection causes an artefactual rise in the apparent concentration of all serum proteins, including enzymes.

8. High protein concentration in serum. This may exert a protective effect on many enzymes, but whether this is of any practical importance in serum enzyme assays is not clear.

In addition to the above general considerations, each enzyme is also subject to the influence of a set of factors

of its own. For instance, individual enzymes may react differently to storage conditions, exposure to light and nature of the diluents, etc. (Thomson, 1962).

2-3

Enzyme assay techniques.

In the present study only creatine kinase activity has been measured in both serum and tissue extracts. All other enzymes studied have been assayed only in serum.

The chemicals used were chiefly obtained from B.D.H. (Poole), Sigma (London) and Boehringer (Lewes). They were of the highest grades commercially available. Glass distilled water was used in all assays. The pH of the buffer solutions was adjusted to the desired value at the temperature of assay (37°C) with a Radiometer Titrator TTT1, using NaOH or HCl solutions.

With the exception of a few kinetic studies all the continuous monitoring of enzyme reactions was carried out on an LKB 8600 automatic reaction rate analyser. All the colorimetric measurements were performed with a Unicam SP1800 spectrophotometer.

In all enzyme reactions involving the NADH/NAD system the nucleotides were prepared fresh. This was to avoid the development of a dehydrogenase inhibitor shown to occur in the  $\beta$ -NADH solution (Fawcett et al., 1961). The exact nature of this inhibitor is unknown. Its effect is greatest on enzymes using NADH as direct substrate, but smaller in coupling enzyme systems where both NADH and the coupling

dehydrogenases are present in excess (Klotzsch and Klotzsch, 1969).

2-3-1

Creatine Kinase.

There are at present at least seven different methods of measuring creatine kinase activity. These include colorimetric, fluorimetric, spectrophotometric and continuous pH titration techniques. Both forward and reverse reactions may be used. These methods are summarised in Fig. 2-2. Of these methods, the only satisfactory continuous monitoring methods are the two coupled-enzyme spectrophotometric methods, since the titrimetric method is not sensitive enough. These, together with two colorimetric methods, were chosen to measure the forward and backward reactions of the enzyme. The formation of MgADP and creatine phosphate from MgATP and creatine was arbitrarily defined as the forward reaction.

It should be noted here that all these four methods, especially the spectrophotometric methods, would be invalidated if the enzyme ATPase was present in the medium.

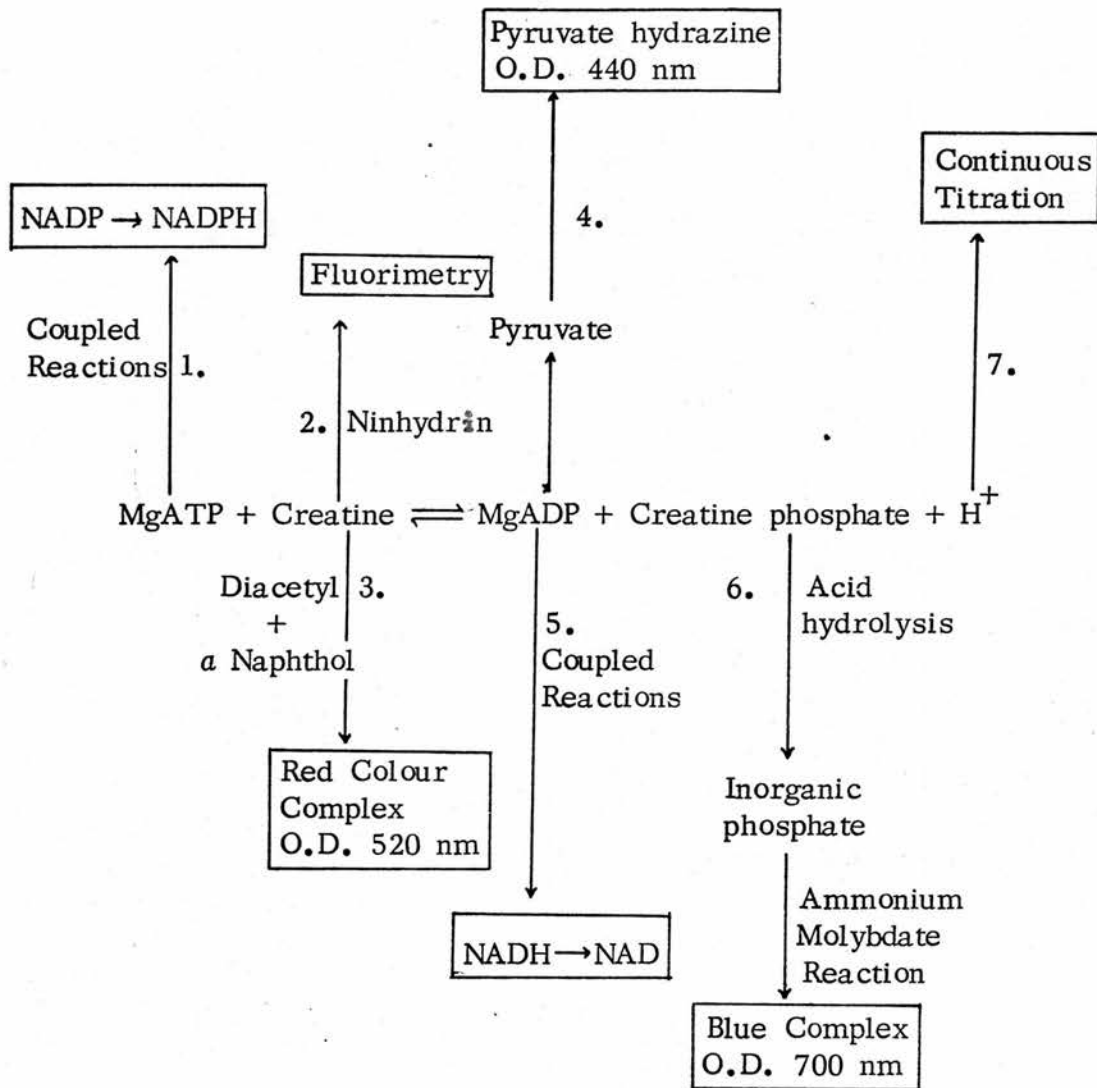
ATPase catalyses the reaction:



ATPase therefore interferes with any methods that base the determination of creatine kinase activity on measurement of ATP, ADP or inorganic phosphate. This is more of a problem with tissue preparations than with serum, as ATPase is seldom present in detectable quantities in human serum.

Figure 2-2

Methods of Measuring Creatine Kinase Activity

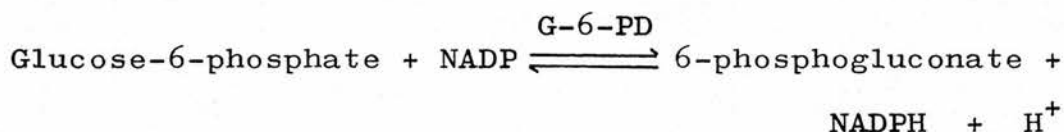
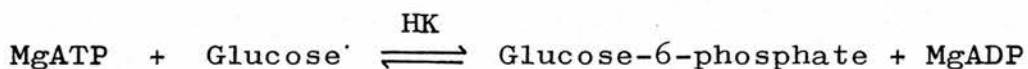
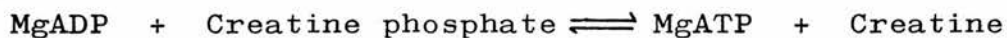


1. Oliver (1955); Rosalki (1967)
2. Conn and Anido (1966)
3. Hughes (1962)
4. Nuttall and Wedin (1966)
5. Tanzer and Gilvarg (1959)
6. Kuby (1954); Okinawa et al. (1964)
7. Cho et al. (1960)

2-3-1-1 Continuous-monitoring method: backward reaction

(Oliver, 1954; Rosalki, 1967).

Principle. This measures the rate of ATP formation in the backward reaction by two coupled enzyme reactions.



When the substrate glucose, coenzyme NADP and coupling enzymes, hexokinase (HK) and glucose-6-phosphate dehydrogenase (G-6-PD) are present in excess, the increase in absorbance of NADPH at 340nm is directly proportional to the rate of ATP production, which in turn is proportional to creatine kinase activity.

Reagents. Both manually prepared reagents and a commercial automatic analysis kit (Boehringer, Lewes) were used for this method. The working reagents in the analysis kit and manually prepared assay systems were as follows:-

<u>Solution 1</u>	<u>*Manually prepared</u>	<u>Analysis kit</u>
Triethanolamine		
buffer pH 7.0	-	100 mmol/l
Tris/HCl pH 7.0	50 mmol/l	-
Magnesium acetate	10 mmol/l	10 mmol/l
ADP	1 mmol/l	1 mmol/l
Glutathione (reduced)	-	9 mmol/l
β-mercaptoethanol	5 mmol/l	-



<u>Solution 1</u>	<u>*Manually prepared</u>	<u>Analysis Kit</u>
Glucose	20 mmol/l	20 mmol/l
NADP	0.6 mmol/l	0.6 mmol/l
Hexokinase	1.5 IU/ml (25°C)	1.5 IU/ml (25°C)
G-6-PD	1.5 IU/ml (25°C)	1.5 IU/ml (25°C)
AMP	-	10 mmol/l

#### Solution 2

Creatine phosphate	10 mmol/l	35 mmol/l
<u>Total assay volume</u>	1 ml	1.1 ml

\* reagent concentrations and assay conditions were varied in certain instances to suit the experiment.

Thiols were added in order to activate and stabilise the activity of creatine kinase (Section 5). As myokinase cannot be excluded from serum samples, AMP was added to inhibit this enzyme (Rosalki, 1967); at the concentration used, AMP was only slightly inhibitory to creatine kinase. As the manual procedure contained no AMP, it was used only for studying the kinetic properties of creatine kinase in purified tissue preparations where myokinase was shown to be absent.

Procedure. 25  $\mu$ l of serum sample was pre-incubated with 1.0 ml assay solution for 15 min at 37°C before the reaction was started with Solution 2. The reaction rate at 340 nm was followed for 2 min.

Assessment of method. A short lag phase ranging from 0.5 to 1.0 min was observed whether the creatine kinase was measured in serum or in a purified preparation. This is

characteristic of coupled enzyme reactions. As already mentioned, the duration of this lag phase is related to the amount of coupling enzymes added. The activity of creatine kinase in the assay mixture (volume 1 ml) was seldom over 30 mIU at 37°C, and the activity of the coupling enzymes was at least 3000 IU/l (namely 100 times higher). The lag phase of this method is much shorter than the method proposed by Rosalki (1967) in which creatine kinase activity was measured at 30°C using the "Calsul" reagent kit from Calbiochem Ltd.; this took as long as 6 min to achieve maximum velocity.

Using human heart tissue preparations (Section 5), it was found that the method gave a linear relationship with enzyme concentration (Fig. 2-3).

The precision of the method, expressed as standard deviations (S.D.) was obtained by repeated analysis of sera from patients. The S.D. was calculated by the formula

$$\sqrt{\sum d^2 / (n - 1)} \quad \text{where } n = \begin{array}{l} \text{total number of observations} \\ d = \text{difference between the} \\ \text{duplicates} \end{array}$$

The standard deviations and coefficients of variation at different levels of creatine kinase activity are listed in Table 2-1. It is seen that at low creatine kinase activity, the precision is poorer.

The commercially available Solution 1 was stated by the manufacturers to be stable for 3 days after reconstitution. In general, this was confirmed by experiments in

Figure 2-3

The Rosalki Method (1967): Correlation between Enzyme Activity  
and Enzyme Concentration

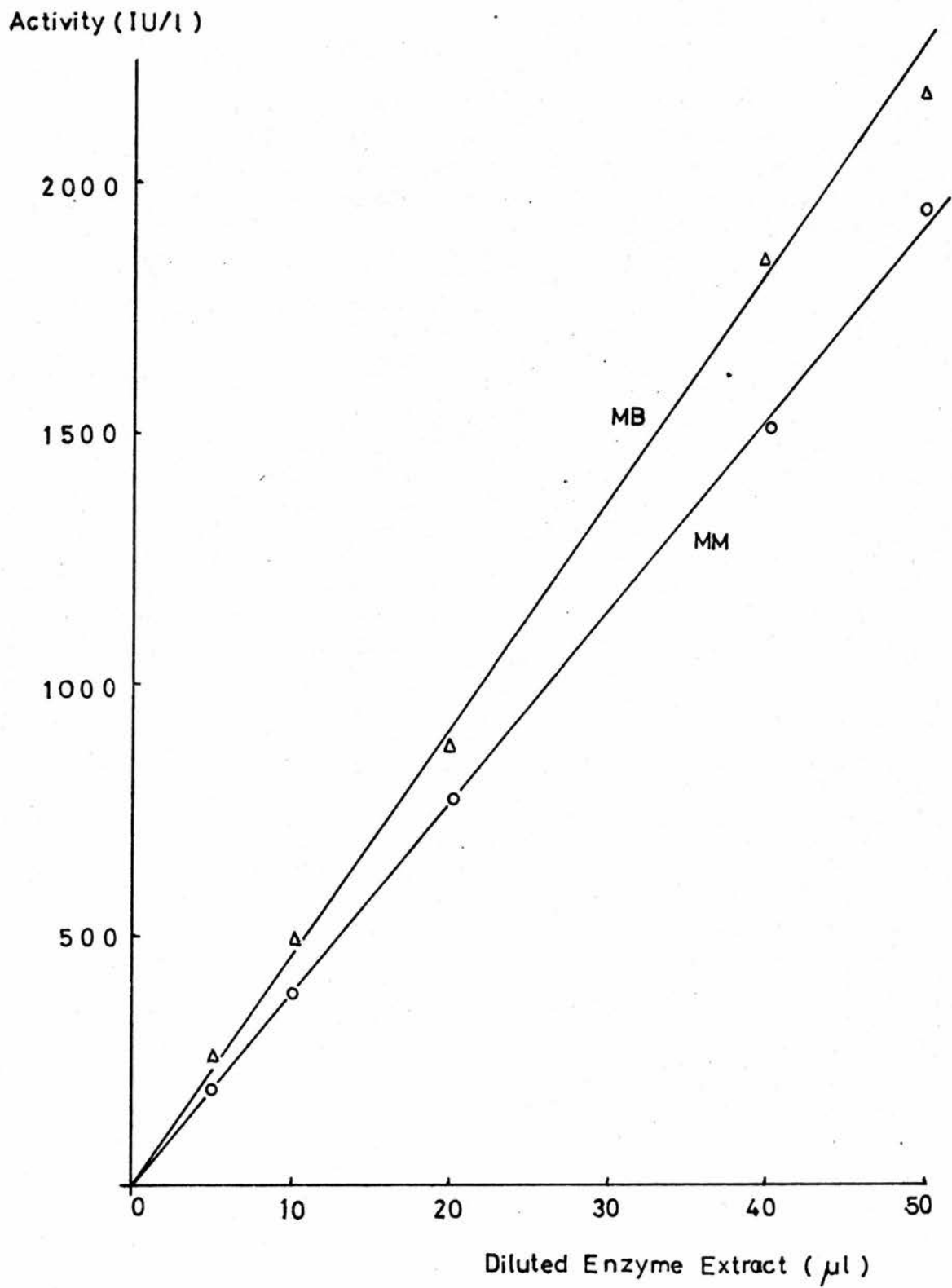


Table 2-1

Precisions of Some Methods Measuring Creatine Kinase Activity

Levels of Activity	*Rosalki (1967)	Tanzer & Gilvarg (1959)	Hughes (1962)
$\bar{x}$ IU/l	52	65	60
S.D.(n)	6.4 (14)	6.3 (36)	15.2 (50)
C. V.	12 %	9.7 %	25 %
$\bar{x}$ IU/l	152	167	180
S.D.(n)	7.9 (14)	7.6 (46)	23.3 (56)
C. V.	5.2 %	4.5 %	12.9 %
$\bar{x}$ IU/l	555	460	412
S.D.(n)	27.8 (14)	21.3 (28)	39 (18)
C. V.	5 %	4.6 %	9.5%
$\bar{x}$ IU/l	3360	-	-
S.D.(n)	134.7 (13)	-	-
C. V.	4 %		

$\bar{x}$  = mean activity

S.D. = standard deviation

C. V. = coefficient of variation

n = number of data obtained

\* = in form of a reagent kit

Serum samples were used for the Rosalki method while purified enzyme solutions were used for the other methods.

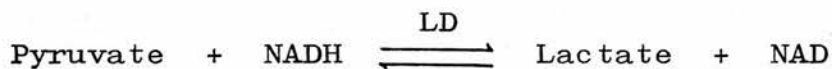
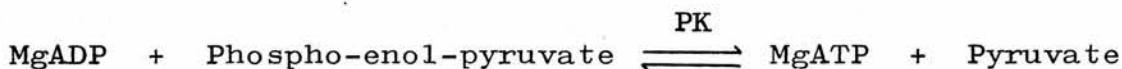
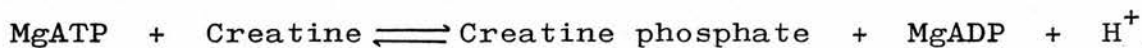
Details of analysis were described in the text.

which sera from patients were analysed on successive days (Fig. 2-10) but there were occasions, especially with high activity specimens, where freshly constituted reagents gave rise to higher activities. Presumably these changes were related to partial inactivation of the coupling enzymes or oxidation of reduced glutathione. In the present investigation, Solution 1 has been discarded if not used within 24 hours of reconstitution.

2-3-1-2 Continuous-monitoring method - forward reaction

(Tanzer and Gilvarg, 1959).

Principle. This measures the rate of ADP production in the forward reaction. The ADP formed is coupled to the formation of NAD monitored by a decrease in absorbance at 340 nm as follows:



PK = pyruvate kinase

LD = lactate dehydrogenase

Reagents. The following mixtures were prepared:

<u>Solution 1</u>	<u>Final concentration</u>
ATP	1.0 mmol/l
Magnesium acetate	2.0 mmol/l
$\beta$ -mercaptoethanol	5.0 mmol/l
Phospho-enol-pyruvate	1.5 mmol/l
Pyruvate kinase	2 IU/ml at pH 9
Lactate dehydrogenase	2 IU/ml at pH 9
$\beta$ -NADH	0.36 mmol/l
Glycine/NaOH pH 9.0	50 mmol/l

Solution 2

Creatine 20 mmol/l

Enzyme sample 25-50  $\mu$ l

Total assay volume 1.0 ml

Procedure. 0.85-0.875 ml of Solution 1 was pipetted into a cuvette and pre-incubated for 15 min at 37°C. The reaction was started with 0.1 ml Solution 2. The reaction was followed for 2-3 min by observing the change of absorbance at 340 nm.

Assessment of method. A lag phase of 1.0 - 1.5 min was observed. The linear velocity obtained after the lag phase was taken as a close approximation of the real initial velocity.

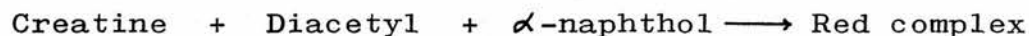
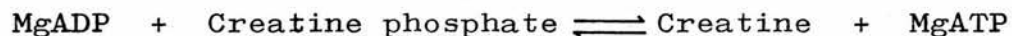
Using tissue preparations, it was found that the activity was linearly related to enzyme concentration only up to a limit, beyond this the curves flattened. This, however,

was not observed in the method of Rosalki (1967) presumably because there a much more dilute enzyme preparation was used. The amount of enzyme and hence activity used in the present method was therefore limited to the linear region of the graph (Fig. 2-4).

The precision of the method was calculated as in method 1 (Table 2-1).

2-3-1-3 Colorimetric-method: backward reaction  
(Hughes, 1962).

This is a colorimetric assay measuring the formation of creatine during the backward direction. The creatine reacts with diacetyl and  $\alpha$ -naphthol to form a red colour complex in an alkaline medium:



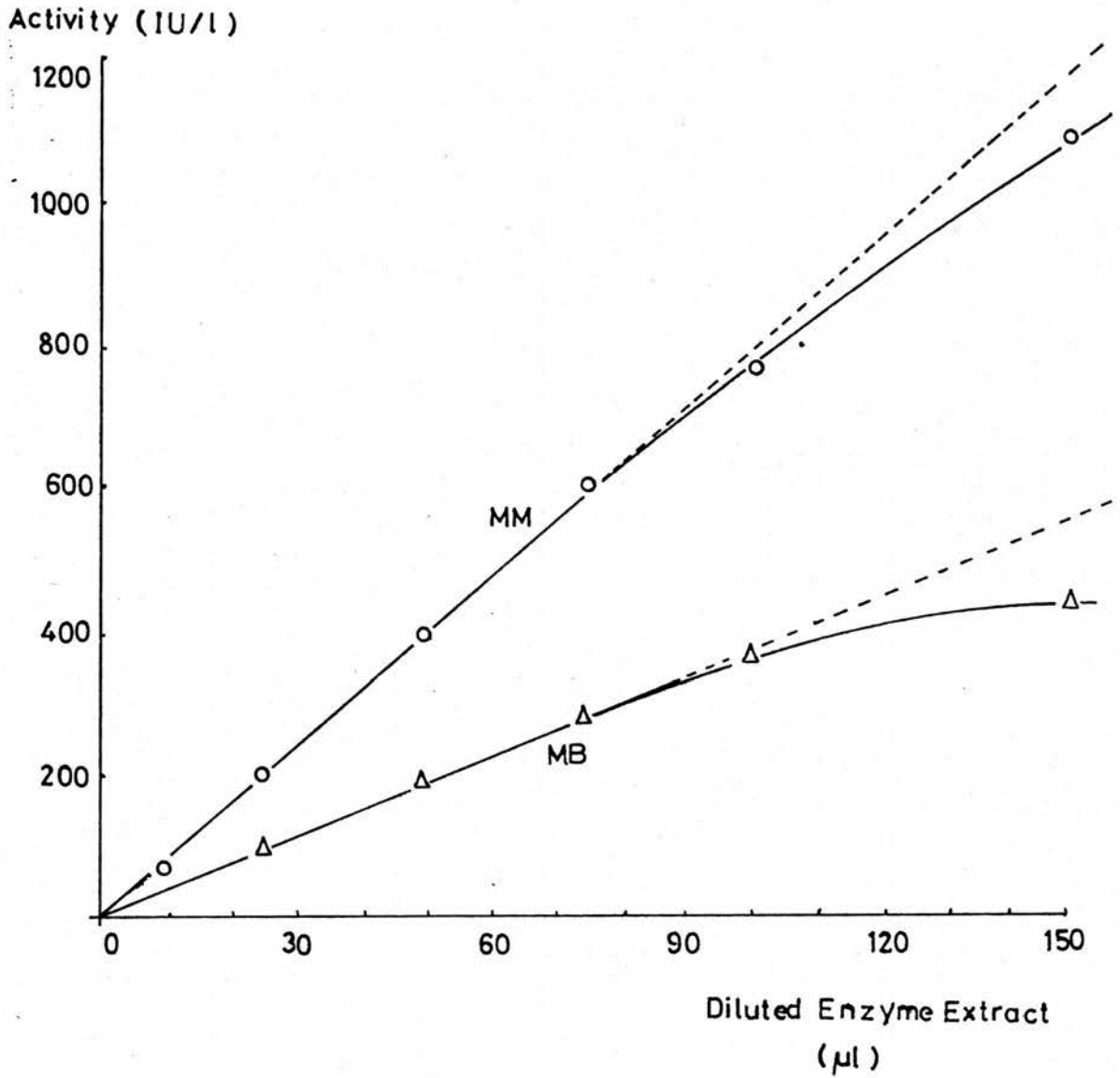
The red complex absorbs maximally at 520 nm.

Reagents. The substrate concentrations in the assay solution (with some modifications in certain experiments) were as follows:

<u>Solution 1</u>	<u>Final concentration</u>
Tris/HCl pH 7.0	50 mmol/l
Magnesium acetate	5 mmol/l
ADP	1 mmol/l
$\beta$ -mercaptoethanol	5 mmol/l

Figure 2-4

The Method of Tanzer and Gilvarg (1959): Correlation between Enzyme Activity and Enzyme Concentration



○ MM isoenzyme with original extract diluted 7.5 times.

Δ MB isoenzyme with original extract diluted 6.0 times.



Solution 2

Creatine phosphate 10 mmol/l

Enzyme solution 50  $\mu$ l

(suitably diluted)

Total assay volume 0.5 ml

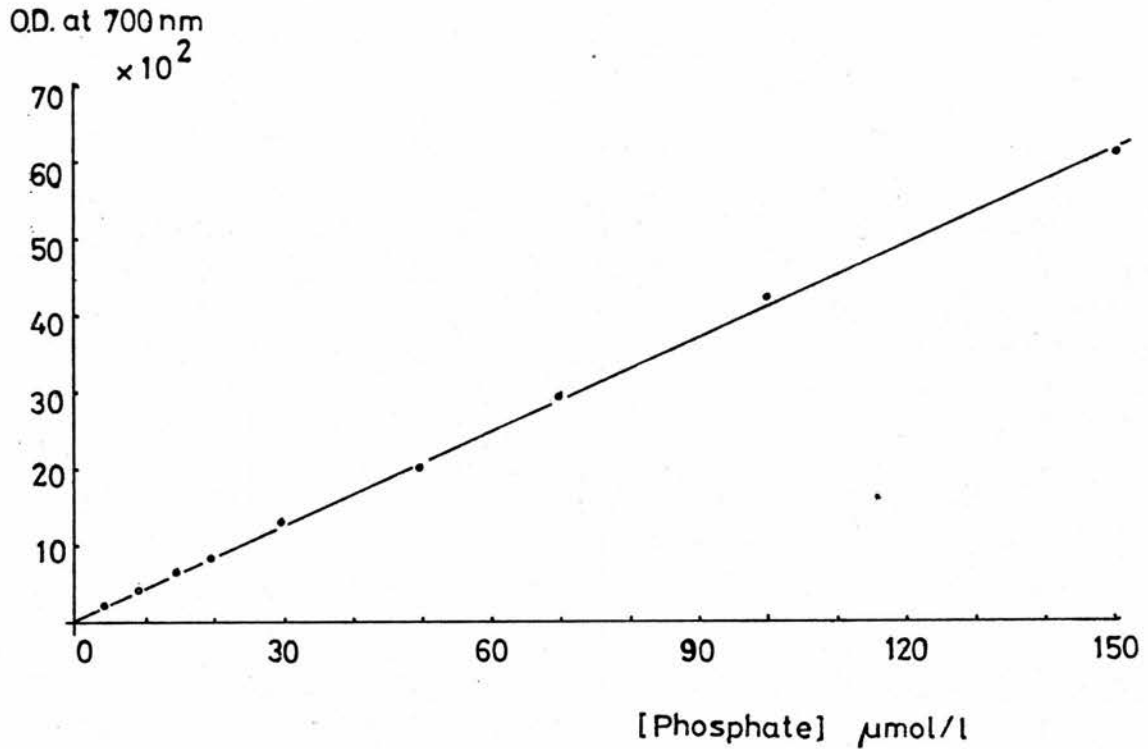
Colour-developing solutions:

- a. 30 mmol/l p-hydroxymercuribenzoate
- b. 60 g/l  $\text{Ba}(\text{OH})_2$
- c. 50 g/l  $\text{ZnSO}_4$
- d. 16 g/l  $\alpha$ -naphthol, freshly made up in 1.5 mol/l NaOH and 1.2 mol/l  $\text{Na}_2\text{CO}_3$
- e. Diacetyl, 1:2000 dilution in water made up freshly

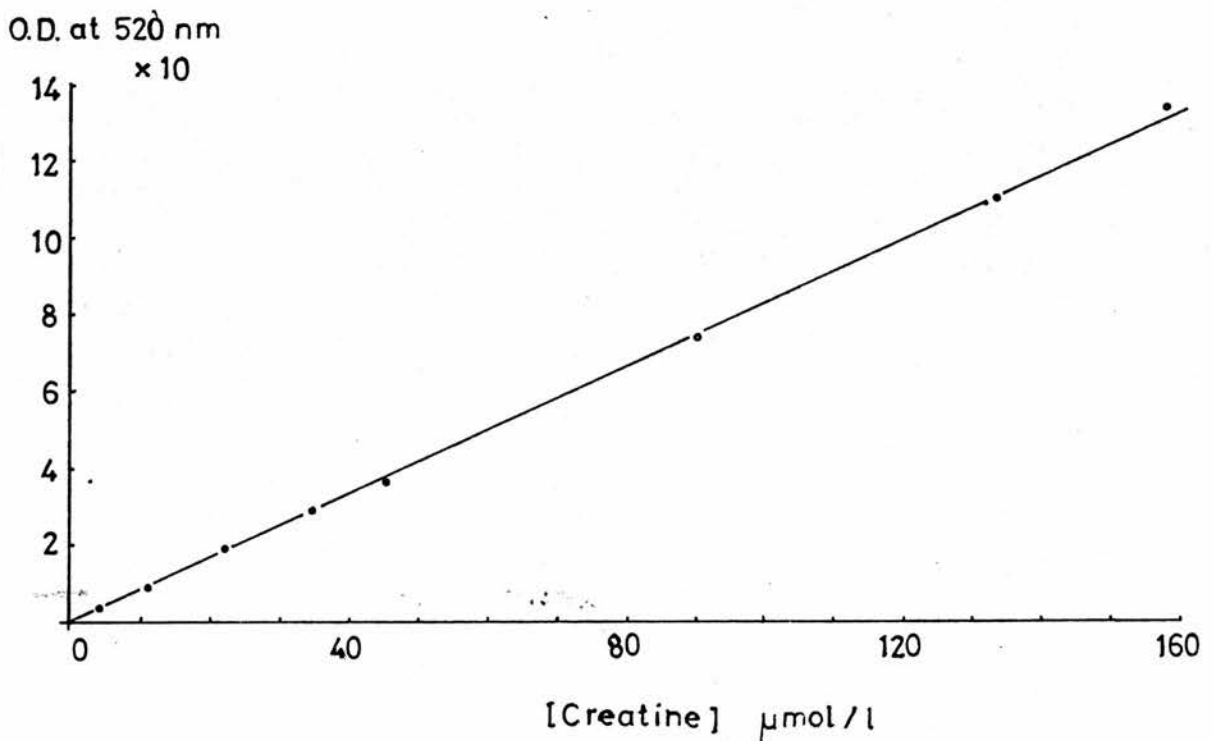
Procedure. The reaction mixture (Solution 1) was pre-incubated in a water bath for 15 min at  $37^\circ\text{C}$  to achieve thermal equilibrium. The reaction was started with Solution 2 and allowed to proceed for 15 min. It was stopped by the addition of 0.5 ml of Solution a. The mixture was then deproteinised by introducing 0.5 ml of Solutions b and c in that order. The precipitate was centrifuged and 1.0 ml of the supernatant was transferred to a fresh tube to which were added 2.5 ml Solution d, 0.5 ml Solution e and 6 ml of water in that order. The tubes were shaken between additions. They were put in a  $37^\circ\text{C}$  water bath for 1 hour, after which the colour in each tube was read at 520 nm against a blank omitting the enzyme solution, and converted to enzyme activity by reading off from a standard curve (Fig. 2-5).

Figure 2-5

a. The Kuby Method (1954) : Standard Curve for the Absorbance of Inorganic Phosphate



b. The Hughes Method (1962): Standard Curve for the Absorbance of Creatine



The protein precipitation step was omitted when purified enzyme preparations were used.

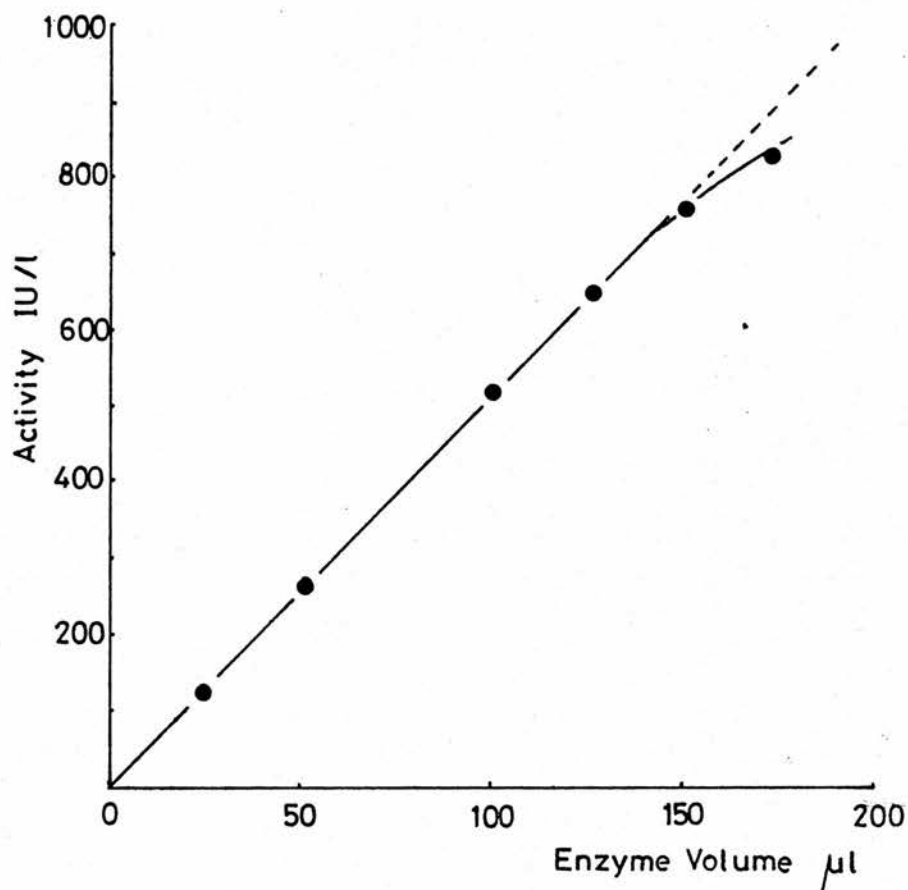
There was at least one blank in each batch, omitting the enzyme or introducing it after Solution a.

Assessment of method. This method is essentially a 2-point assay method. There is no check that the reaction is linear. However, if no more than 20% of the substrate is consumed in the course of the reaction, only minor variations (largely arising from variations in the use of substrate concentrations) should be encountered, and it was not considered necessary to follow the course of each reaction by withdrawing samples at timed intervals from the reaction mixture. This, in practical terms, means that if the enzyme concentration used is not higher than those shown in Fig. 2-6a, in a 15 min incubation period, the measured velocity should be a good approximation to initial velocity (Fig. 2-6b). The precision of this method was assessed at different levels of enzyme activity (Table 2-1).

Apart from the possible danger of non-linearity of the reaction, the creatine colour development may also be a source of error in this method as it is sensitive to minor variations in the experimental conditions. A long incubation period (1 hour at 37°C) for the colour development is needed to minimise this effect (Hughes, 1962). Moreover, the creatine colour development is inhibited by thiols which are essential to ensure full activation of creatine kinase. Therefore, thiols must be removed before colour development, and this is done by adding p-hydroxy-mercuri-

Figure 2-6

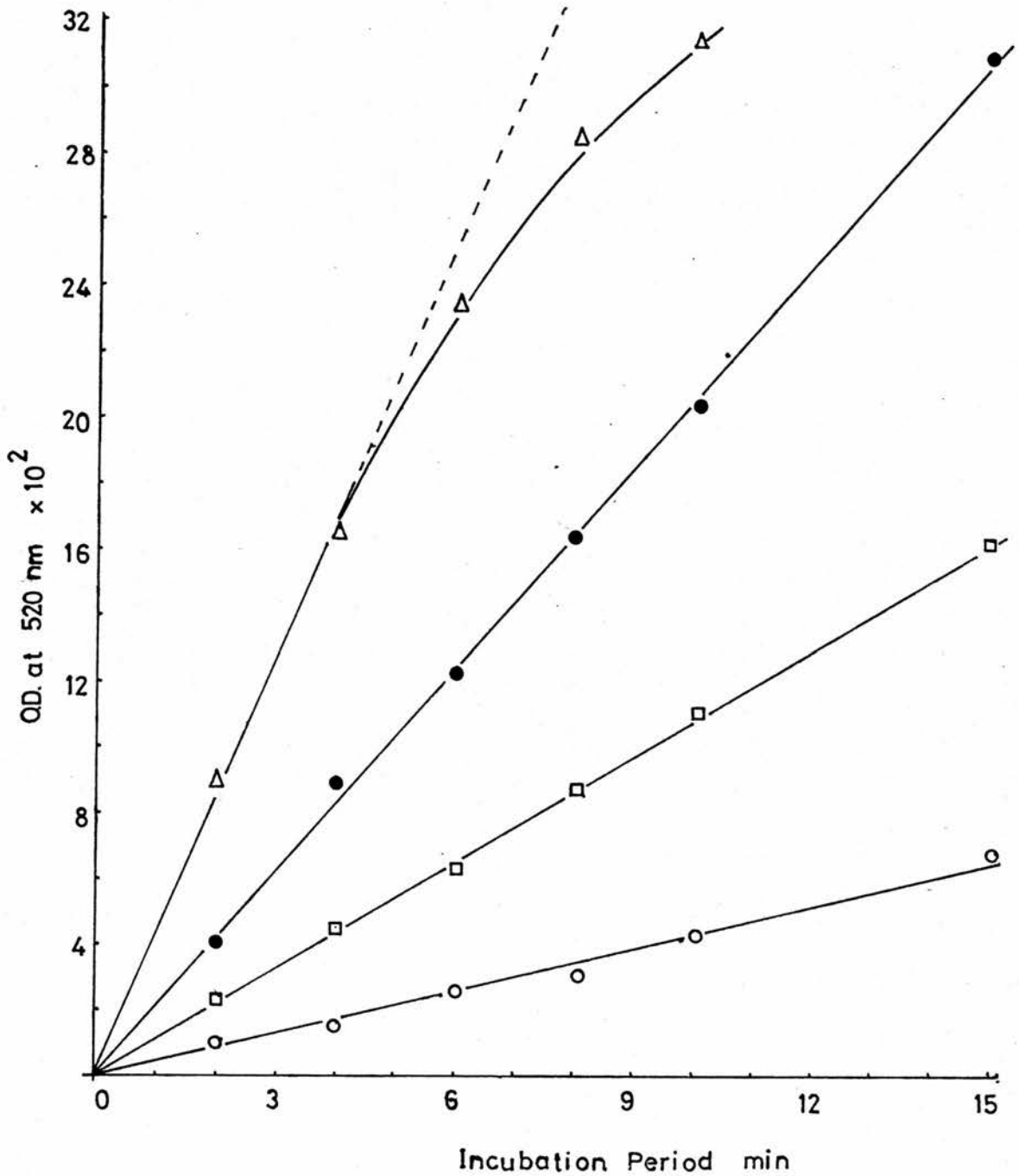
a. The Hughes Method: Correlation between Enzyme Activity and Enzyme Concentration



The original Isoenzyme (MM) preparation was diluted 200 fold. Enzyme activity was measured after 15 min incubation period at 37°C. Linear relationship between enzyme activity and enzyme concentration was observed within the sample volume range 0 - 150  $\mu$ l.

Figure 2-6

b. The Hughes Method : Time Course of the Reaction of Creatine Kinase (MM Isoenzyme)



The original enzyme preparation was diluted 200 fold :

○ 25  $\mu$ l      □ 50  $\mu$ l      ● 100  $\mu$ l      Δ 175  $\mu$ l

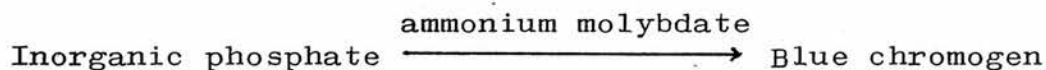
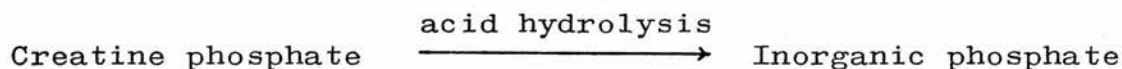
benzoate which also serves to stop the enzyme reaction.

Also, absorbance measurements should be made soon after the colour has been developed, since the colour fades over a period of a few hours.

#### 2-3 -1-4 Colorimetric method: forward reaction

(Kuby, 1954; Okinawa et al., 1964).

Principle . This method measures the amount of creatine phosphate formed from the forward reaction. The creatine phosphate is hydrolysed with acid and the amount of inorganic phosphate liberated is determined with the classical ammonium molybdate reaction. The blue colour complex formed has a maximum absorption at 700 nm.



Reagents Unless stated otherwise, the reagent concentrations were as follows:

<u>Solution 1</u>	<u>Final concentration</u>
ATP	1.5 mmol/l
Magnesium acetate	3.0 mmol/l
$\beta$ -mercaptoethanol	5.0 mmol/l
Glycine/NaOH pH 9.0	50 mmol/l
<u>Solution 2</u>	
Creatine	20 mmol/l

<u>Enzyme solution</u>	50 $\mu$ l
<u>Total assay volume</u>	1.0 ml

Colour developing solutions:

- a. 20g/l ascorbic acid and 100 g/l trichloroacetic acid
- b. 10g/l ammonium molybdate in water
- c. 20g/l sodium citrate + 20g/l sodium arsenite in 20g/l acetic acid.

Procedure. The reaction was started by adding 0.2 ml Solution 2 to 0.75 ml Solution 1 after the solutions had been equilibrated at 37°C with the enzyme solution. The reaction was allowed to proceed for 15 min, at the end of which it was stopped by adding 1.5 ml of Solution a. After standing for 30 min at room temperature (25°C), 0.5 ml Solution b was added followed by 1.0 ml Solution c. The mixture was thoroughly shaken between each addition. The blue colour took about 15 min to develop (Baginski *et al.*, 1967). The colour was read and converted to enzyme activity units with a standard graph (Fig. 2-5 ).

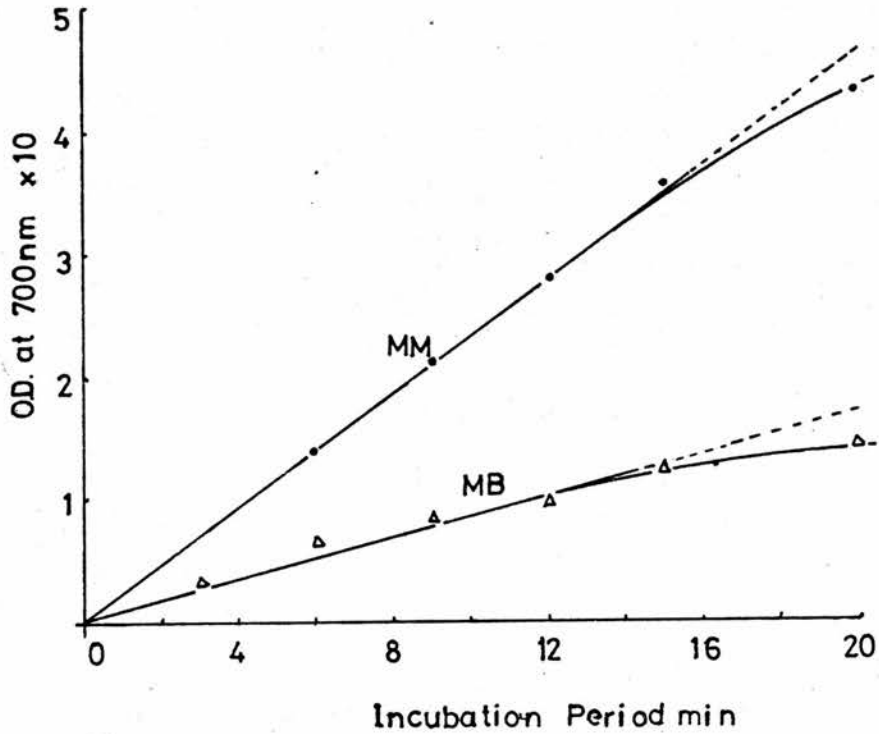
Blanks were prepared for each concentration of the adenine nucleotide substrate as it contained traces of inorganic phosphate and was slightly (1-3%) hydrolysed during the complete hydrolysis of creatine phosphate.

Assessment of method. Being a 2-point assay this method has the same limitations as Method 3. Therefore, the time-course of the reaction was followed with an active enzyme sample (Fig. 2-7a). From these results an incubation period of 15 min was considered suitable for the concentration of enzyme used in this study. The inorganic phosphate

Figure 2-7

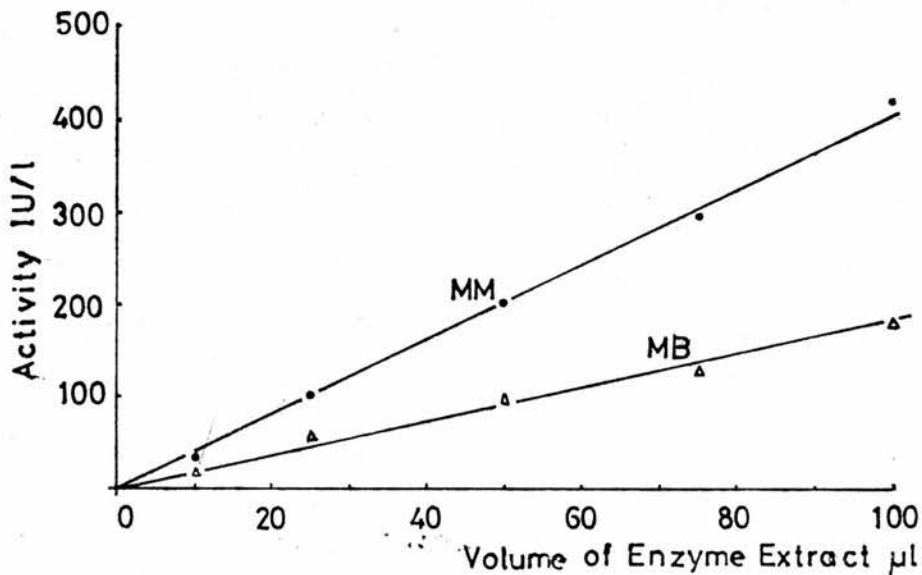
The Kuby Method (1954)

a. Time Course of the Reaction of Creatine Kinase



The two isoenzyme preparations were each diluted 10 fold, using 50  $\mu$ l for the MM isoenzyme assay and 75  $\mu$ l for the MB.

b. Correlation between Enzyme Activity and Enzyme Concentration



The isoenzyme extracts were 10-fold dilutions of their respective original preparations.



measured after hydrolysis was found to be proportional to enzyme concentration (Fig. 2-7b).

Not enough data were available for the assessment of precision of the method. The limited number of data, however, did suggest that it was of the same magnitude as the colorimetric assay of Hughes (1962).

The major drawback of this technique is the presence of inorganic phosphate in the blank. This reduces the sensitivity of the assay and also necessitates accurate blank correction. In the original method of Fiske and Subbarow (1925) there were additional blank problems caused by the continuing slow hydrolysis of organic phosphates occurring after the addition of ammonium molybdate, the liberated phosphates contributing to the final colour developed. In the present study this problem has been overcome by adding arsenite to exhaust all the excess ammonium molybdate after the ammonium molybdate had complexed with inorganic phosphate (Baginski et al., 1967). Any subsequent formation of inorganic phosphate arising from the hydrolysis of labile organic phosphates would no longer contribute to the colour development.

#### 2-3-1-5 Selection of a method for measuring serum creatine kinase activity.

A method had to be chosen that was suitable for measuring the activity of a large number of serum samples in the clinical study (Section 4). Relevant considerations

included the requirement that the method should preferably be simple and convenient.

From Table 2-1 it is evident that the spectrophotometric assays are more precise than the colorimetric techniques at all levels of enzyme activity. They are more sensitive, and less laborious to perform. Using the LKB 8600 automatic analyser, they require much less time for analysis than the corresponding colorimetric assays.

Both spectrophotometric methods show the same order of precision, but the method of Rosalki (1967) is more sensitive than the method of Tanzer and Gilvarg (1959). The difference in sensitivity is probably attributable to inherent kinetic differences between the backward and forward reactions. The magnitude of this difference was determined by comparing, for the same amount of enzyme, the maximum velocities of both forward and backward reactions using the two spectrophotometric methods of assay that have been studied. With purified isoenzyme preparations, ratios ( $V_{\max}$  backward at pH 7.0/ $V_{\max}$  forward at pH 9.0) of 10.3 and 3.6 were obtained for the MM and MB isoenzymes respectively. These values suggested large differences in the turnover rates of the isoenzymes (especially the MM isoenzyme) between forward and backward reactions when assayed in the conditions described above.

The method of Rosalki, which measures the backward direction was, for the above reasons, considered to be the most suitable of the methods studied here for measuring serum creatine kinase activity. For convenience, the

Boehringer analysis kit was used exclusively for this purpose. The other methods were reserved for the kinetic studies of the creatine kinase isoenzymes (Section 5).

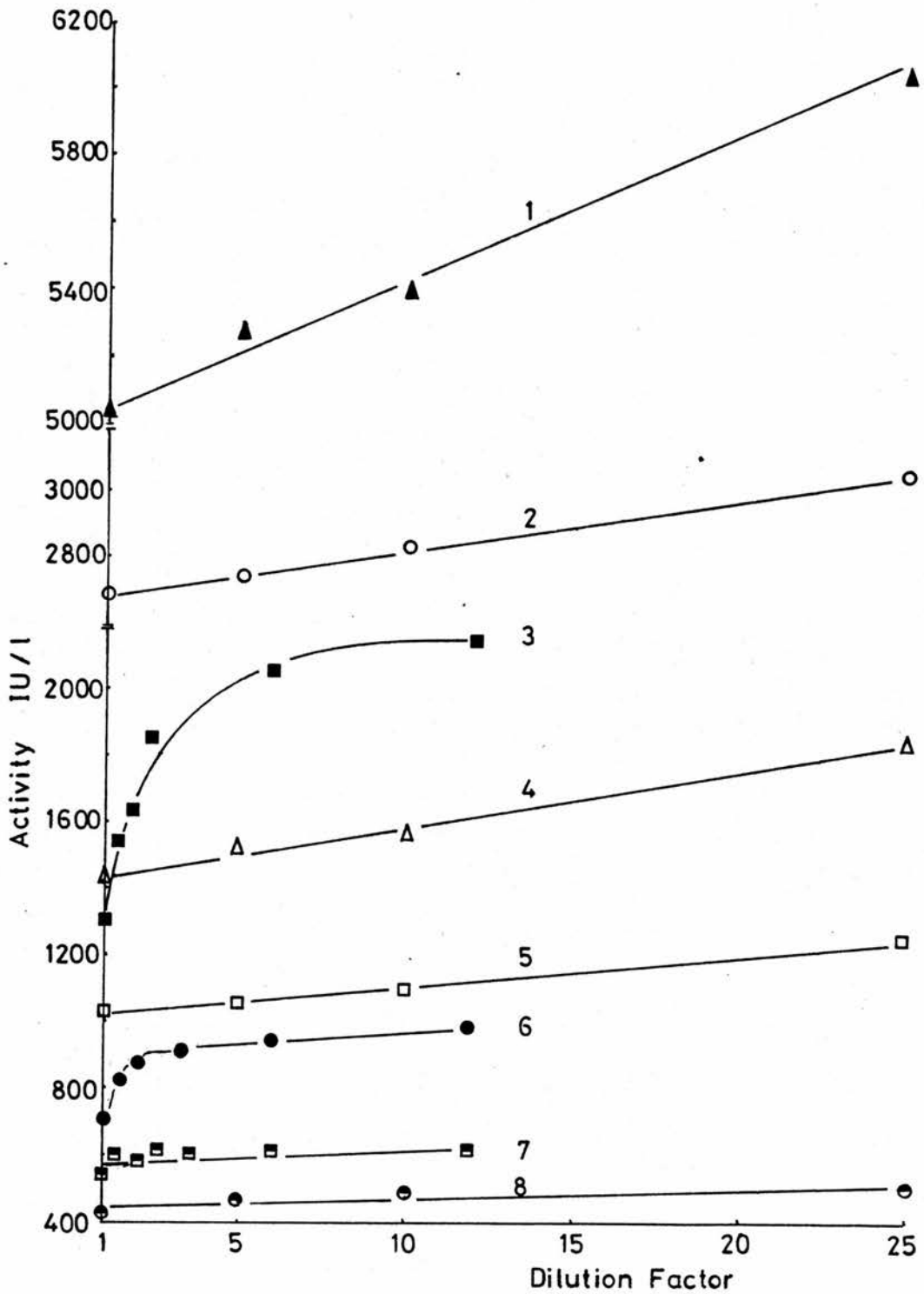
2-3-1-6 Effect of dilution on serum creatine kinase activity.

Although a linear relationship was obtained between activity and enzyme concentration using purified enzyme solutions, this was not the case when serum samples were used (Compare Figs. 2-3 and 2-8). Elevation of activity was observed on diluting serum samples. The exact nature of this activation effect is still unsettled. Craig et al., (1967) reported a progressive increase of activity when serum samples from patients with various pathological conditions were diluted. The dilution effect was observed irrespective of the direction of the reaction or the methods used for measuring enzyme activity. The extent of activation varied with the nature of diluents. The authors recommended the use of heat-inactivated serum as diluent as it gave the least activation among the diluents studied. On the basis of the progressive nature of the activation that occurred on dilution of samples, they argued against the presence of an inhibitor in serum.

To investigate more fully the effect of diluting serum upon the results of enzyme activity measurements serum samples were chosen from 8 different patients with myocardial infarction, covering a wide range of creatine kinase activity.

Figure 2-8

Effect of Dilution with Water on the Activity of Serum Creatine  
Kinase



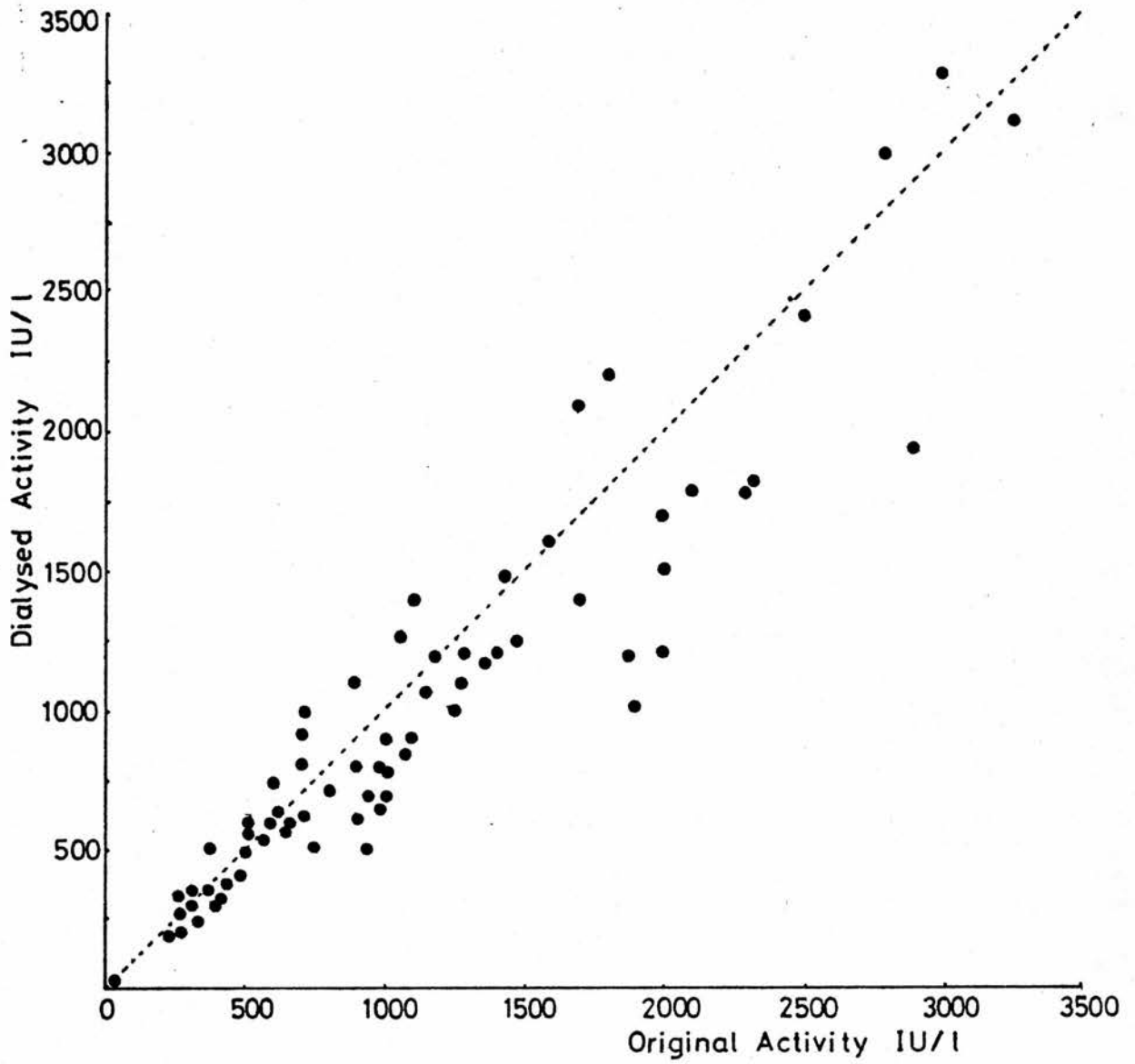
1 - 8 Serum samples

The sera were diluted with distilled water in varying proportions, and creatine kinase activities measured (Fig. 2-8). In contrast to the findings in the above reports, creatine kinase was found to be activated to a relatively small extent, an increase of 1.1 to 1.3 times on a 25-fold dilution, with the exception of Sample 3 in which the activity increased 1.6 times when diluted 12-fold. In those samples showing the most activation (Samples 3 and 6) the rise in activity occurred almost entirely in the initial (up to about 1:5) dilutions with further dilution having little effect. This might be explained by the presence of a "serum inhibitor" the effects of which were removed almost completely by dilution. The rest of the samples showed only a small increase in activity which was linear with dilution in the range studied. So, while some samples did suggest the existence of a serum inhibitor, others did not support the presence of such inhibitors in serum.

The effect of dialysis was studied with a batch of 70 serum samples from patients with myocardial infarction. The specimens were dialysed against 5 mmol/l Tris/HCl buffer pH 8.0 for 24 hours with 3 changes of buffer. Figure 2-9 shows the relationship between activities before and after dialysis. The majority of the samples (i.e. below the 45° line) indicated some loss of activity, probably due to the effect of slight dilution occurring during dialysis. There were a few that showed activation, but in no case was the activation greater than 20 per cent. The many-fold increase and decrease in activity after dialysis reported by Snehalatha

Figure 2-9

Effect of Dialysis on the Activity of Serum Creatine Kinase



et al., (1973) was not observed here. The data from these dialysis experiments thus give no support for the hypothesis that a dialysable serum inhibitor or activator exists.

From these two experiments and the reports in the literature mentioned above it is likely that the effect of diluting serum upon creatine kinase activity not only varies with disease states, but also between individuals. The nature of this effect of dilution is likely to be complex and cannot be explained by any simple theory.

#### 2-3-1-7 Other factors affecting serum creatine kinase activity.

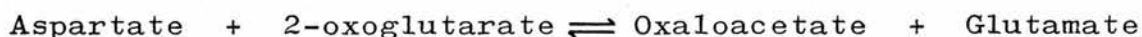
Thiol groups. In the present work 2 common thiols, reduced glutathione and  $\beta$ -mercaptoethanol, were used to activate the enzyme. In the absence of thiol groups the enzyme activity is sensitive to minor changes of assay conditions and rapidly becomes inactivated in serum. Although the exact mechanism of this thiol activation is not certain, the thiol is thought to act by keeping the thiol groups of the enzyme essential to activity in reduced form, and by protecting them from any thiol-blocking reagents.

Storage conditions. These affect the stability of serum creatine kinase. Thomson (1962) suggested storage at 4°C in darkness instead of deep-freezing, as he found that decrease in activity at 4°C was less marked. He also reported a loss of activity of up to 25% when serum samples were exposed to daylight..

The stability of creatine kinase activity in serum from patients with proven myocardial infarction was studied. The samples were stored at 4°C in darkness as recommended. In all samples studied, no significant change of activity was observed over a period of 3 weeks (Fig 2-10). The minor fluctuations in activity can be explained by the lack of precision of the method used.

### 2-3-2 Aspartate aminotransferase in serum.

The enzyme catalyses the reaction



Various methods have been used to measure the activity of this enzyme in the past. The following is a brief summary of some of these techniques:

Chromatographic assay (Awapara and Seale, 1952). This measures the formation of the product, glutamate, by quantitative paper chromatography. The glutamate is eluted from the paper and forms a coloured complex with ninhydrin. Manometric method (Krebs, 1950). The amount of amino acids changed or produced can be measured by using specific amino acid decarboxylases.

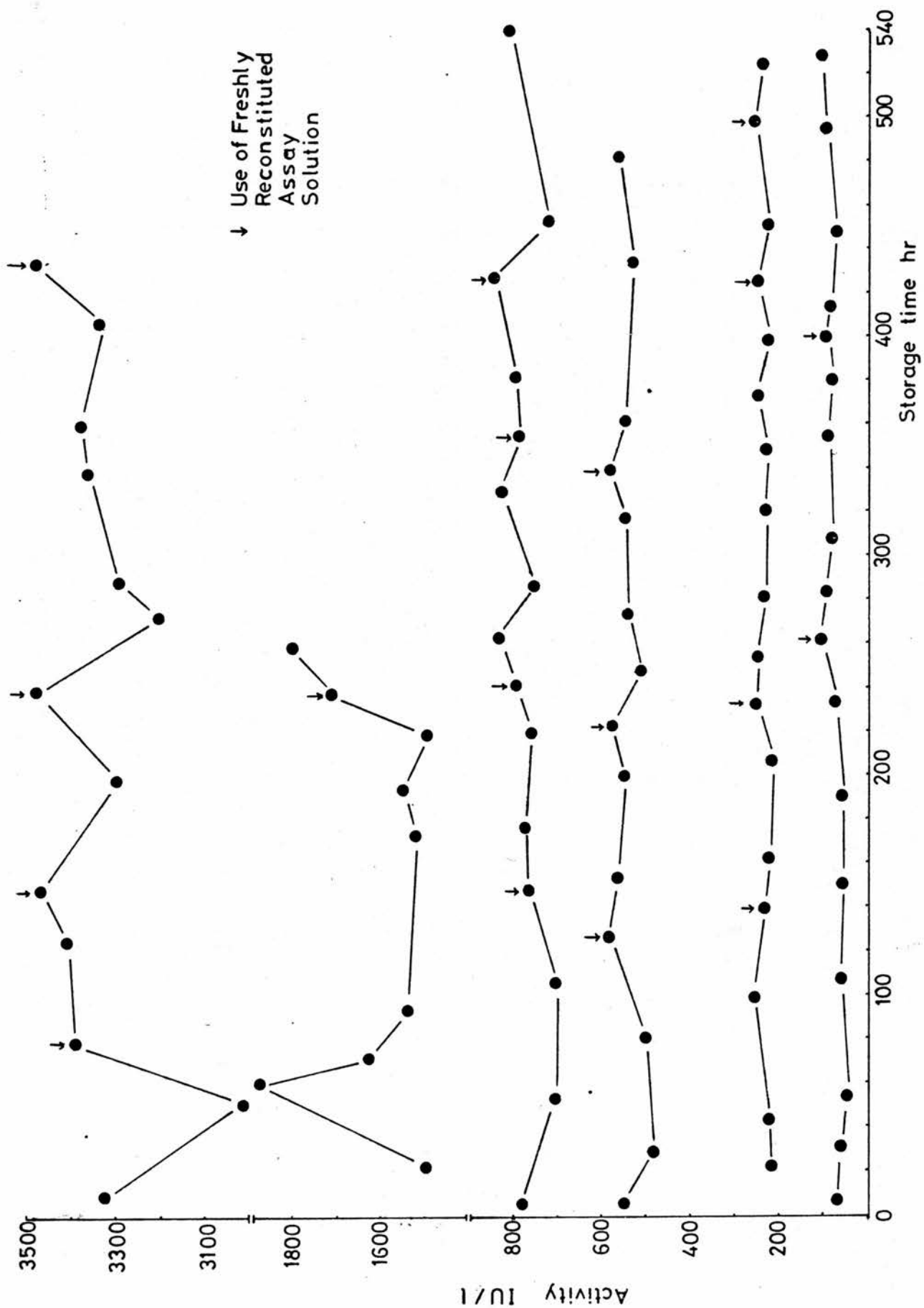
Colorimetric methods. The oxaloacetate formed is converted to pyruvate in the presence of aniline citrate. The pyruvate then reacts with dinitrophenyl hydrazine to form pyruvate-dinitrophenyl hydrazone which is extracted with toluene. The colour intensity is taken as a measure of enzyme activity (Tonhazy *et al.*, 1950). This method has





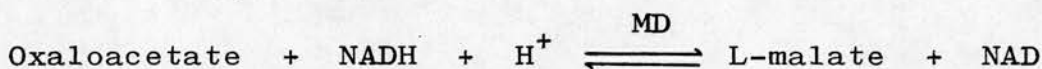
Figure 2-10

Stability of Serum Creatine Kinase Activity on Storage  
at 4°C



been simplified by Reitman and Frankel (1956). Another colorimetric method has been described by Babson et al., (1962), in which the oxaloacetate formed is coupled with a stable diazonium salt. The resulting colour is proportional to the amount of oxaloacetate present and can be measured at 500-550 nm.

Spectrophotometric methods. The high UV absorption of oxaloacetate at 280 nm can be used to follow the transamination reaction (Cammarata and Cohen, 1951), but the instability of oxaloacetate, the relatively low aminotransferase activity in serum, and the high protein concentration in serum make this method unsatisfactory. Another spectrophotometric method developed by Karmen (1955) is widely used. This is a coupled enzyme technique:-



The decrease in absorption of NADH at 340 nm is a measure of the aminotransferase activity provided malate dehydrogenase (MD) and co-enzyme NADH are in excess.

The chromatographic and manometric methods are obsolete, and the colorimetric methods are gradually being superseded by the more precise, more convenient and faster UV kinetic assay in serum activity determinations. In the present study all serum aspartate aminotransferase activities were measured by the method of Karmen (1955) as modified by Henry et al. (1960).

Reagents.

<u>Solution 1</u>	<u>Final concentration</u>
L-aspartate	127 mmol/l
$\beta$ -NADH	0.21 mmol/l
Malate dehydrogenase suspension (Boehringer)	0.8 mmol/l
Potassium phosphate buffer pH 7.0	100 mmol/l
<u>Solution 2</u>	
2-oxoglutarate	5 mmol/l
<u>Serum</u>	100 $\mu$ l
<u>Total assay volume</u>	1.0 ml

Procedure.

0.85 ml volumes of Solution 1 were pipetted into cuvettes and pre-incubated with serum samples at 37°C for about 15 min. The reaction was started by adding 0.05 ml Solution 2. The absorbance change at 340 nm was followed for 1 min.

Assessment of method.

The enzyme requires pyridoxal phosphate as co-enzyme. So far it has been standard laboratory practice to assume that the co-enzyme is present in sufficient quantities in the serum and that no extra amount of the co-enzyme is needed in the assay medium. However, Hamfelt (1966) considered that pyridoxal phosphate should be added to the serum of elderly patients, chronic alcoholics, and patients with thyrotoxicosis or intermittent porphyria. Rej et al.

(1973) recommended the use of 25  $\mu\text{mol/l}$  pyridoxal phosphate in the assay solution, and reported that this gave an average increase of 16% in the enzyme activity.

Side-reactions occur in the assay solution before the reaction is started by the addition of 2-oxoglutarate, as serum contains an appreciable quantity of 2-oxoglutarate. There might therefore be a danger that the concentration of NADH could become sub-optimal as a result of these endogenous reactions. The amount of NADH added for use in the assay was, however, sufficient to offset these reactions in nearly all cases.

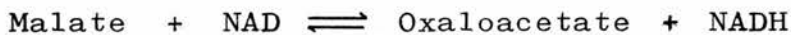
If the coupling enzyme is introduced as an ammonium sulphate suspension, the ammonium ion may react with 2-oxoglutarate in the presence of glutamate dehydrogenase, which is present in serum in some pathological states:



In most cases a pre-incubation period at 37°C for 15 min is long enough for the completion of these side reactions. The between-batch precision of the method in the range up to 50 IU/l was 1.8 IU/l.

### 2-3-3 NAD - dependent malate dehydrogenase .

The enzyme catalysis the reaction



Many early methods used oxaloacetate as substrate and followed the reaction from right to left because the position of the equilibrium at pH 7.4 is favourable in this

direction (Siegel and Bing, 1956). The main technical difficulty in measuring activity in this direction is the instability of oxaloacetate, which decomposes to pyruvate in aqueous solution. Because lactate dehydrogenase activity in serum is usually many times more active than malate dehydrogenase, this newly formed pyruvate would contribute significantly to the measured reaction velocity. To overcome this difficulty the oxaloacetate can be produced enzymatically by inclusion of purified aspartate aminotransferase and L-aspartate in the assay solution. The reaction is then started by adding an excess of 2-oxoglutarate. Alternatively, the reaction can be measured in the opposite direction using as starting substrate malate, which is more stable than oxaloactate. In the present study a spectrophotometric method using malate as starting substrate was developed.

Reagents.

<u>Solution 1</u>	<u>Final concentration</u>
NAD	3 mmol/l
Diethanolamine	
buffer pH 9.2	100 mmol/l
<u>Solution 2</u>	
L-malate	25 mmol/l
<u>Serum</u>	100 $\mu$ l
<u>Total assay volume</u>	1.0 ml

Procedure. 0.85 ml volumes of Solution 1 were pipetted into cuvettes and pre-incubated with serum samples at room temperature for 1 hour followed by 15 min at 37°C. The

reaction was started by adding 50  $\mu$ l L-malate solution. The increase in absorbance at 340 nm was followed for 1 min.

#### Assessment of method

Choice of Buffer. Among the different buffers studied, triethanolamine, 2-amino-2-hydroxy-methyl 1:3 propandiol, tris/HCl, glycine/NaOH, borate, diethanolamine and diethylbarbiturate, diethanolamine gave the highest activity. With this buffer the optimal pH at 37°C was found to be in the region of 9.2. Serum activity of malate dehydrogenase was little affected by the strength of the buffer in the range 10 to 900 mmol/l. 100 mmol/l was adopted as the working buffer concentration.

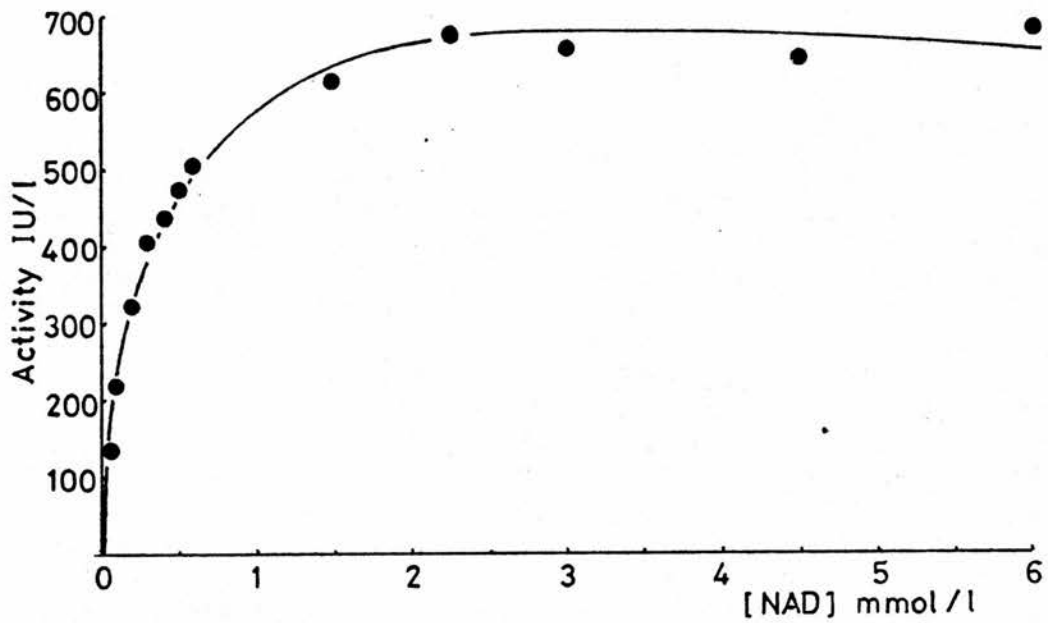
Substrate concentrations. The substrate affinities for malate and NAD were investigated with five randomly chosen active serum samples. They all showed that malate at high concentration was inhibitory. No such inhibition was observed for NAD (Fig. 2-11a and b). Working concentrations of 25 mmol/l and 3 mmol/l were chosen for malate and NAD respectively. Magnesium ions were found to activate the serum enzyme slightly (0-8%), but the extent of activation was not thought to be large enough to warrant its inclusion in the reaction mixture.

Owing to the presence of endogenous lactate and lactate dehydrogenase in serum, the addition of NAD to the mixture led to its spontaneous reduction. This side-reaction had to be allowed to proceed until all lactate was exhausted. A standing period of one hour at room temperature before the

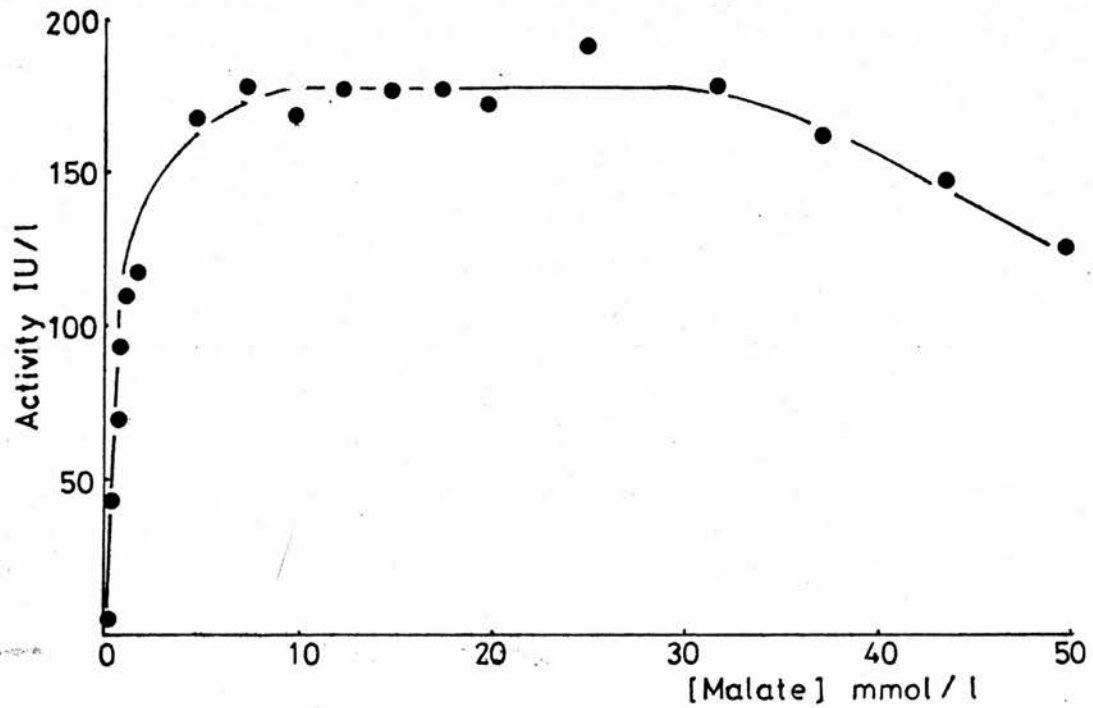
Figure 2-11

Assay of Malate Dehydrogenase in Serum:

a. Variation of Enzyme Activity with NAD Concentration



b. Variation of Enzyme Activity with Concentration of L-malate



reaction was started with malate was found to be sufficient.

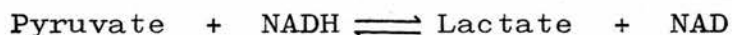
This technique gave activity data proportional to enzyme concentration, as shown in Fig. 2-11c. Its precision, obtained by duplicate analysis of serum samples, is listed in Table 2-2. The correlation between this technique and that of Siegel and Bing (1956) is shown in Fig. 2-11d.

#### 2-3-4      Assay of miscellaneous enzymes.

In the course of the study activities of some other enzymes in serum were also measured. They were the anodal isoenzymes of lactate dehydrogenase, alanine aminotransferase and glutamate dehydrogenase.

##### Urea-stable lactate dehydrogenase.

Lactate dehydrogenase activity is measured in the presence of urea, which inhibits the activity of the isoenzymes of lactate dehydrogenase to a varying extent.



This measures principally the anodal isoenzymes of lactate dehydrogenase and is used as a test in the diagnosis of myocardial infarction.

The method of Brydon and Smith (1973) was used.

<u>Solution 1</u>	<u>Final Concentration</u>
$\beta$ -NADH	0.18 mmol/l
Urea	1.8 mmol/l
Phosphate buffer pH 7.5	100 mmol/l



Figure 2-11

Assay of Malate Dehydrogenase in Serum:

c. Correlation between Enzyme Activity and Enzyme Concentration

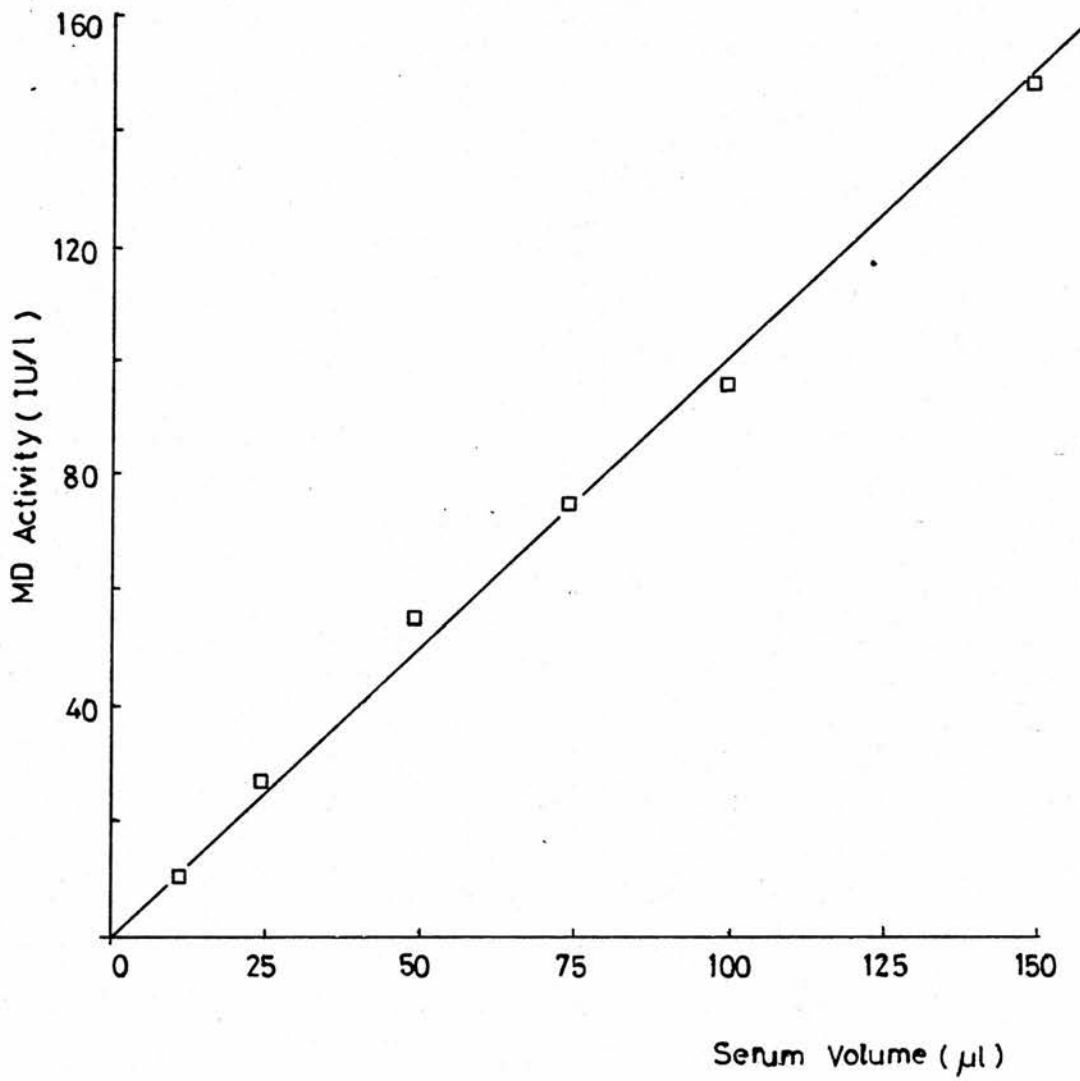


Figure 2-11

Assay of Malate Dehydrogenase Activity in Serum:-

- d. Correlation between the Present Technique and that of Siegel and Bing (1956) Using Oxaloacetate as Substrate

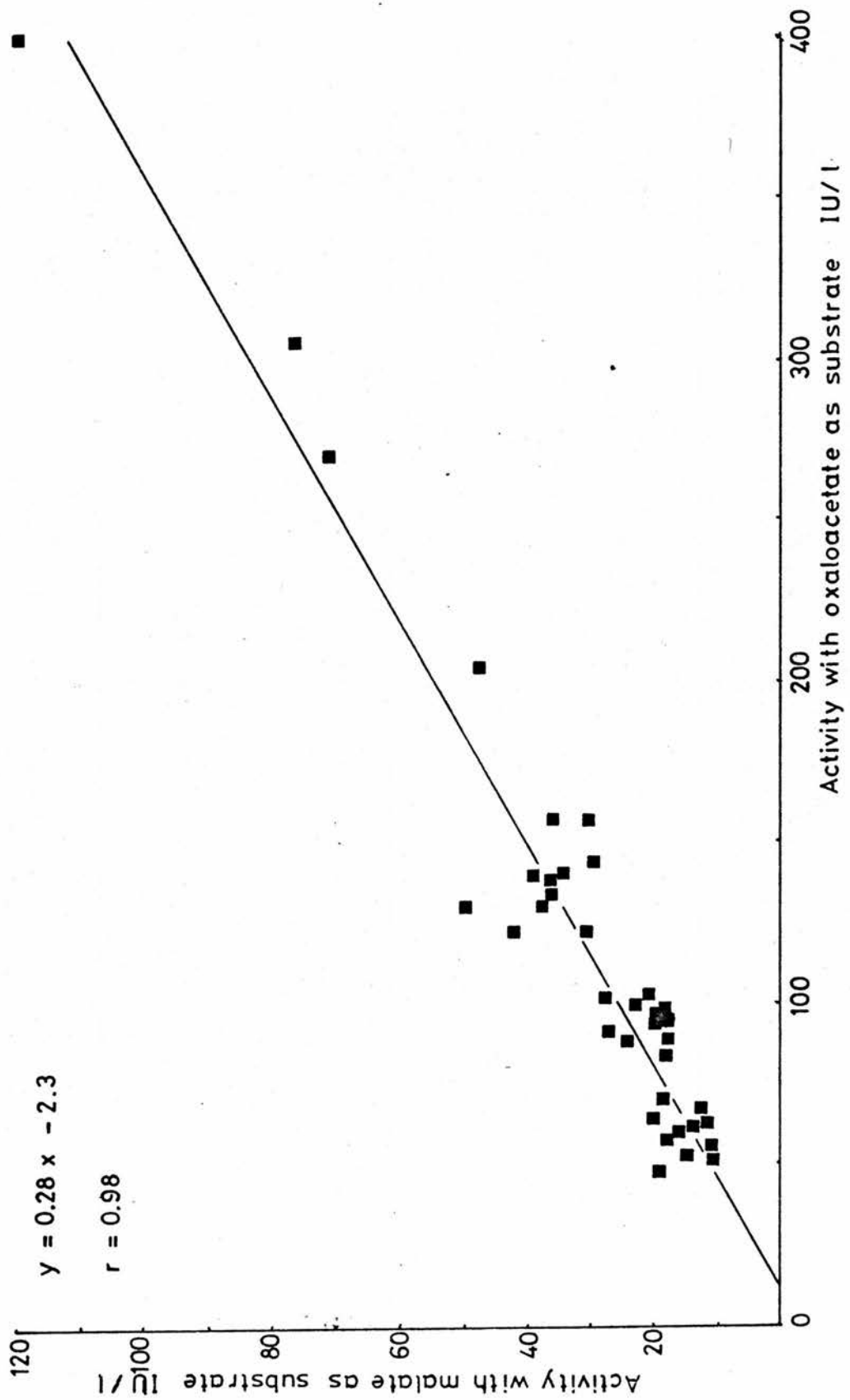


Table 2-2

Precision of the Malate Dehydrogenase Assay Using  
Malate as Substrate

Range IU/l	Mean IU/l	S.D. IU/l	C.V.
0 - 30 (23)	18	3.0	16.6%
30 - 100(11)	44	4.0	9.1%
100 - 750 (6)	287	6.6	2.3%

( ) = number of pairs of data

S.D. = standard deviation

C.V. = coefficient of variation

<u>Solution 2</u>	<u>Final Concentration</u>
Sodium pyruvate	
(starting substrate)	3 mmol/l
<u>Serum</u>	10 $\mu$ l
<u>Total assay volume</u>	1.0 ml

The reaction rate was monitored at 340 nm on an LKB 8600 reaction rate analyser for 1 min for each sample. The between-batch precision of the method was 20 IU/l in the range up to 500 IU/l.

Alanine aminotransferase (Karmen, 1955; Henry et al., 1960).

The activity was measured by a coupled enzyme reaction.

Alanine + 2-oxoglutarate  $\rightleftharpoons$  Glutamate + Pyruvate

Pyruvate + NADH  $\xrightleftharpoons{\text{LD}}$  Lactate + NAD

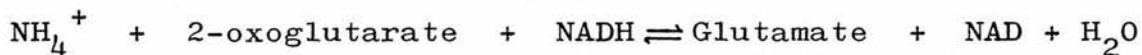
When lactate dehydrogenase (LD) and NADH are present in excess, the decrease in absorbance of NADH at 340 nm is in index of alanine aminotransferase activity.

<u>Solution 1</u>	<u>Final Concentration</u>
L-alanine	425 mmol/l
$\beta$ -NADH	0.19 mmol/l
Lactate dehydrogenase	1-2 IU/ml
Phosphate buffer pH 7.4	100 mmol/l

<u>Solution 2</u>	
2-oxoglutarate	5 mmol/l
(starting substrate)	
<u>Serum</u>	100 $\mu$ l
<u>Total assay volume</u>	1.0 ml

The reaction rate was measured for 1 min at 340 nm after a pre-incubation period of 15 min at 37°C. The between-batch precision in the range up to 50 IU/l was 2 IU/l.

Glutamate dehydrogenase (Ellis and Goldberg, 1972).



The assay solution contained:

<u>Solution 1</u>	<u>Final concentration</u>
$\beta$ -NADH	0.19 mmol/l
2-oxoglutarate	10 mmol/l
ADP	0.95 mmol/l
Triethanolamine	
buffer pH 7.4	50 mmol/l
<u>Solution 2</u>	
Ammonium sulphate	50 mmol/l
(starting substrate)	
<u>Serum</u>	200 $\mu$ l
<u>Total assay volume</u>	1.0 ml

The reaction was followed at 340 nm at 37°C for 2 min. Ammonium sulphate was used to start the reaction in preference to 2-oxoglutarate, as originally described by Ellis and Goldberg (1972), because the endogenous L-aspartate would react with 2-oxoglutarate in the presence of serum aspartate aminotransferase. Although L-aspartate is present in only very small amounts in serum, if 2-oxoglutarate was to be used as starting substrate, this reaction would add to the measured reaction velocity.

2-4

Quality control.

In view of the large number of serum enzyme samples that had to be measured over a long period (about 10 months), rigorous quality control of the assay methods was needed. The performance of all determinations in duplicate was not considered practicable as it would not only have doubled the time and expense of all enzyme assays, but the very pressure of repetitive work could have become a major factor itself contributing to unsatisfactory quality of results. The repeat analysis of a limited number of serum samples that had already been determined in the previous batch was, however, routinely employed. The use of such repeat specimens from patients had the advantage over the use of pooled serum controls that the specimens from patients had been stored for much shorter periods and hence had had less time in which to deteriorate. This method of quality control was used to monitor the short-term day-to-day precision of the assay methods. It was of less value in assuring the long-term stability of a method. For this a freeze-dried commercially available enzyme control material, Precipath E (Boehringer Corporation, Lewes), was used routinely.

SECTION 3

THE RELEASE OF CYTOPLASMIC AND MITOCHONDRIAL  
ENZYMES IN ACUTE MYOCARDIAL INFARCTION

This section and the next describe the enzyme findings in a group of patients admitted to the Coronary Care Unit of the Royal Infirmary, Edinburgh, with suspected myocardial infarction. In the present section the principles underlying the release of enzymes after cell injury are discussed. The patterns of release of certain mitochondrial enzymes, namely glutamate dehydrogenase (GLDH) and the mitochondrial isoenzymes of aspartate aminotransferase (mAST) and malate dehydrogenase (mMD), have been studied and compared with those of the cytoplasmic isoenzymes of aspartate aminotransferase (cAST) and malate dehydrogenase (cMD).

### 3-1 Theoretical considerations.

#### 3-1-1 Plasma enzymes in health.

Many enzymes have been detected in blood plasma. They can in general be classified into two groups, those that have metabolic functions in plasma and those that do not. The first group includes all those enzymes involved in blood coagulation, fibrinolysis and the complement system. The second group includes a diversity of enzymes which are either secreted by cells or released into the circulation as a result of the normal "wear and tear" of the body tissue.

In healthy individuals the activities of most enzymes in plasma stay more or less constant, indicating that the



rates of release into and removal from plasma are equal.

However, some variation in these "normal" levels may occur among individuals due to the influence of a number of factors, including:-

1. Race and other genetic factors (Rucknagel and Neel, 1961).
2. Social Class. For example, it has been found that there is a correlation between social class and plasma alkaline phosphatase activity (Goldberg and Winfield, 1974).
3. Physiological State. The reports of Wilding et al. (1972) and Goldberg and Winfield (1974) showed that many "normal" enzyme levels are affected by the age, weight and sex of the individual. The state of physical fitness and exercise may also have an effect on the plasma activity of AST, creatine kinase (CK), lactate dehydrogenase (LD) and MD (Nuttall and Jones, 1968; Halonen and Konttinen, 1962). Normal muscular activity may produce high levels in some young ("enzyme-labile") subjects (Griffiths, 1966). Nutritional and psychic states are also a determining factor.
4. Miscellaneous Factors. Climate, occupation, living standard and even season of the year may, to a smaller extent, affect plasma enzyme activities (Richterich, 1968).

The handling of blood specimens before enzyme assay may affect the activity measured. For example, it has been reported that the chilling of samples of clotted blood may cause an irreversible efflux of enzymes from erythrocytes without producing haemolysis (Sweetin and Thomson, 1973).

Most of the available evidence indicates that the range of variation in plasma enzyme activity in the same individual

is less than the range of variation seen between individuals, even if the population is homogeneous with regard to age, sex and race. Although theoretically desirable to use an individual as his own source of reference data, this is usually impracticable. Therefore, it is customary to evaluate the results of plasma enzyme tests in relation to reference values obtained from the healthy population, matched if possible for age, sex and any other factor known to be important in determining plasma activity in health. The reference range of values obtained from such a population often approximate either to a normal distribution or to a log-normal distribution. In either case, it is possible using appropriate parametric methods to evaluate a range, between the 2.5 percentile and the 97.5 percentile, which will include 95% of the results obtained from a healthy population. Alternatively, if it is not wished to make any assumptions about the distribution of the results, non-parametric methods may be used (Reed et al., 1971). Unless stated otherwise, reference ranges derived in this study will be based on 95% confidence limits obtained by the appropriate parametric method.

### 3-1-2 The release of enzymes into the circulation in disease.

In healthy individuals only minor fluctuations of plasma enzyme levels are seen in most cases. In disease states, however, there may be a significant change in the activity of plasma enzymes. With a few exceptions, where the

activity of certain plasma enzymes is decreased (e.g. decreased cholinesterase in liver disease, and decreased alkaline phosphatase in hypophosphatasia), the majority of diseases where plasma enzymes are of value in diagnosis involve an increase in plasma enzyme activity. This increase may be due to a change in the permeability of cell membranes in the affected tissue or to cell necrosis as result of infection, trauma or anoxia. To a smaller extent, the rise in plasma enzyme activity may also be caused by impaired excretion of the enzyme due to obstruction of excretory pathways (e.g. raised plasma amylase in pancreatic duct obstruction).

Zierler (1958) studied the permeability of muscle cells to aldolase and concluded that aldolase efflux was increased by a high external  $K^+$  concentration, anoxia or deficiency of glucose. He suggested that cell-membrane depolarisation might contribute to the release of enzymes. Dreyfus et al. (1958) studied the mechanism of release of aldolase in progressive muscular dystrophy, and considered that increase in cell permeability was caused by anoxia, shortened life-span of the muscle cell and the general reaction of the whole body to stress. Thomson et al. (1960) also studied the release of enzymes in muscular dystrophy and concluded that the primary cause for the release was a change of cell membrane permeability, not cell necrosis, as they argued that fewer enzymes were released in neurogenic muscular atrophy in which there is more extensive muscular damage than in muscular dystrophy. The release of hormones can also have

a profound effect on the release of enzymes. For example, adrenocorticotrophic hormone and cortisone increase plasma aldolase activity by 200-1000% in the rat and the rabbit (Schapira, 1954).

Henley et al. (1958) compared the release of AST and ALT (alanine aminotransferase) in liver slices and found that alkaline pH favoured the release of ALT while its substrate alanine promoted its retention, thus suggesting that the presence of substrate in cells might affect the release of its enzyme. Innerfield (1960) stated that water and electrolyte changes in the inflamed tissue caused efflux of proteolytic enzymes. Recent work on human leukocytes and rat lymphocytes (Wilkinson and Robinson, 1974) suggested that the integrity of the cell membrane was related to the concentration of ATP in the cell. The presence of phospholipases A and C in inflammatory fluids might also play a part in liberating intracellular enzymes (Robinson and Wilkinson, 1973). The release of these intracellular enzymes is, among other things, determined by their shape and molecular weight. In principle, the smaller the molecular weight, the more likely the enzyme is to diffuse out of the cell (Schmidt and Schmidt, 1967). In addition to the molecular weight, the intracellular location and the binding properties of the enzyme molecule to substrates and cell membranes are important.

The release of enzymes from heart tissue in myocardial infarction is believed to be due to ischaemia. Human heart tissue is rich in many enzymes especially MD, LD,

glyceraldehyde-3-phosphate dehydrogenase, AST, L-glycerol-3-phosphate dehydrogenase, CK, aldolase and isocitrate dehydrogenase in decreasing order of abundance (Schmidt and Schmidt, 1960b). In the infarcted area of heart tissue there is a rapid decline in most glycolytic and oxidative enzymes (Gudbjarnason et al., 1967). The enzymes lost are believed to be released from the cell, and a portion of these enzymes enters the circulation. Most enzymes found to be depleted in the infarcted myocardium can be shown to exhibit raised activity in the blood plasma with the notable exception of isocitrate dehydrogenase. The failure to detect a rise of isocitrate dehydrogenase in plasma has been attributed to the rapid clearance rate of the enzyme from plasma (Strandjord et al., 1959), and to the thermal instability of the enzyme (Campbell and Moss, 1962).

The mechanisms controlling release of enzymes from cells are still incompletely understood. It is, however, fairly certain that cell necrosis is not the only cause for the release of enzymes in myocardial infarction. Reversible changes in membrane permeability without cell death, increased intracellular enzyme synthesis in the injured cell, and the release from non muscle-cells around the infarcted area (as a result of change of cellular composition due to damage) may all contribute significantly to the increase in plasma enzyme activity.

3-1-3

Fate of the released enzymes.

Little is known about this subject. At one time it was thought that biliary excretion might play a major role in the removal of enzymes from plasma (Armstrong et al., 1934). Later work, however, showed that this was not so for many enzymes. Hepatectomy had little effect on the clearance of plasma aminotransferases (Cleeve, 1962). Work on aspartate aminotransferase (Dunn et al., 1958) and aldolase (Sibley, 1958) also showed that biliary excretion played little or no part in the clearance of these enzymes.

The renal excretion of plasma enzymes has been investigated for many years. Rosalki and Wilkinson (1959) reported that, except for some digestive enzymes, most plasma enzymes of use in diagnosis were detected only in traces in urine. Subsequently, Kemp and Laursen (1960), Amador et al. (1965) and Appert et al. (1968) showed that renal excretion could not account for the clearance of lactate dehydrogenase, alkaline phosphatase, aspartate aminotransferase, alanine aminotransferase or amylase from plasma, except in renal damage. Removal via the saliva (Cornish and Posen, 1968) and the gastrointestinal tract (Fleisher and Wakim, 1968) were also shown to be unimportant routes of elimination.

Fleisher and Wakim (1963a; 1963b) and Wakim and Fleisher (1963a; 1963b) showed that the reticuloendothelial system was a major site for the clearance of aspartate aminotransferase isoenzymes, alanine aminotransferase and lactate dehydrogenase from plasma. They showed that

blockage of the reticuloendothelial system by zymogen caused a decrease in the clearance rate of injected enzymes. However, the clearance of alkaline phosphatase remained unchanged when the reticuloendothelial system was blocked (Mahy and Rowson, 1965).

Posen (1970) suggested that at least three mechanisms were involved in the clearance of plasma enzymes:

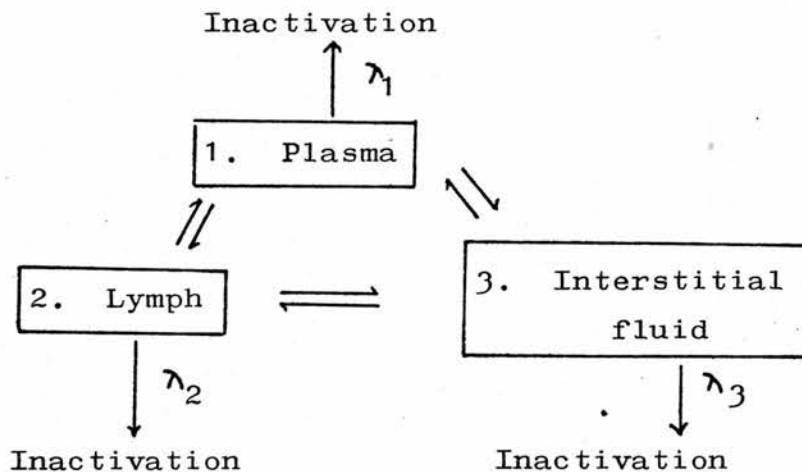
1. Rapid binding of the enzymes at sites near the intra-vascular compartment. This mechanism seems able to be saturated and applies especially to denatured protein.
2. A mixing phase which is mainly a dilution effect between intra- and extra-vascular compartments.
3. Irreversible denaturation by exo- and endopeptidases and by non-enzymic mechanisms.

Other removal mechanisms may also exist. For example, it is possible that enzyme inhibitors may be produced in plasma in response to high plasma enzyme levels in certain cases (Wilkinson, 1962). There is also the possibility that the plasma enzymes might be incorporated into a variety of cells. Indeed, uptake of enzymes by cells has been observed (Warburg et al., 1954).

The clearance rate of most plasma enzymes can be divided into at least two phases, an initial rapid and a subsequent slow phase. The rapid phase is due mainly to the equilibration of the enzyme between the intra- and extravascular spaces; and the slow phase represents the inactivation or denaturation of the circulating enzyme. Fleisher and Wakim (1963a) suggested a three compartment



model to describe the decay in activity of plasma enzymes. The compartments are the plasma space, the lymph space and the interstitial fluid space:-



The clearance rate of a plasma enzyme can be approximated by a mathematical expression:

$$A = a_1 e^{-\lambda_1 t} + a_2 e^{-\lambda_2 t} + a_3 e^{-\lambda_3 t} + A_{\infty}$$

where  $A$  = activity of the plasma enzyme at time  $t$ .

$A_{\infty}$  = activity of the plasma enzyme at steady state.

$a_{1-3}$  = arbitrary constants.

$\lambda_{1-3}$  = inactivation constants of the plasma enzyme in compartments 1-3.

Values of  $\lambda$  may be obtained by plotting  $\log (A - A_{\infty})$  against time.

#### 3-1-4 Origin of plasma enzymes - organ specificity and enzyme pattern.

In most disease states, many enzymes are released into



the circulation, but not all of them are suitable for diagnostic purposes. The rise in activity of the enzyme in plasma must be high enough to permit a sharp distinction between normal plasma activity and activity in the pathological state under consideration. It is also important that the enzyme should be easily related to its tissue of origin to indicate the site of damage. For routine diagnostic measurements, which are likely to be requested frequently, it is highly desirable that the technique of measurement should be simple to perform.

Organs and tissues, depending on their functions, have different enzyme compositions. The differences may be qualitative, but in most cases they are only quantitative. One way of tracing the origin of raised plasma enzymes is to study the relative activities of several plasma enzymes. As most tissues have a fairly distinct enzyme composition, if a definable enzyme pattern is found in plasma, this suggests that a particular tissue is involved in the disease. Such an approach is, nevertheless, liable to misinterpretation as a result of pattern distortion after the enzymes have entered the circulation. The causes for such distortion may be different rates of release, diffusion and clearance of the enzymes, changes in cellular composition, and changes in intracellular enzyme pattern of the tissue due to damage. The enzyme pattern may also be distorted by the release of various enzymes from more than one tissue of the body.

Another method of determining the origin of plasma

enzymes is the measurement of isoenzyme activities in plasma. The present section is concerned with the release of subcellular-specific isoenzymes, a comparative study on the liberation of cytoplasmic (cAST and cMD) and mitochondrial (mAST, mMD and GLDH) enzymes from the damaged myocardium in patients. The use of isoenzymes in the diagnosis of myocardial infarction has been discussed in Section 1, and Section 4 deals with the release pattern and tissue specificity of the MM and MB isoenzymes of creatine kinase.

### 3-1-5 Release of mitochondrial enzymes in myocardial infarction.

Soon after the discovery of the diagnostic value of plasma AST in myocardial infarction by LaDue et al. (1954), the clinical value of many other plasma enzymes was investigated. Consequently, LD and later CK were found to be useful in the diagnosis of the disease. The clinical value of MD in myocardial infarction has also been established although the test is seldom used; Yakulis and co-workers (1962) reported, however, that there was no change in the isoenzyme pattern of MD in the sera of patients after myocardial infarction and suggested that such studies had little value in diagnosis. Glutamate dehydrogenase has not been found to be of value in the diagnosis of acute myocardial infarction, but, has proved a useful index of damage in certain types of liver disorders (see below); it is possible that a similar use for the test could be developed in patients with myocardial infarction.

Subcellular fractionation studies have shown that mAST, mMD and GLDH are all located in the inner membrane matrix fraction of the mitochondrion (Addink et al., 1972; Lardy and Ferguson, 1969). The order of abundance of AST, MD and GLDH in human heart tissue is MD ( $1 \times 10^4$  units/g protein) > AST ( $1 \times 10^3$  units/g protein) > GLDH (20 units/g protein) (Schmidt and Schmidt, 1960). Between 40 and 68% of the total AST is mitochondrial (Bodansky et al., 1968). The corresponding percentage for MD in human heart is less well documented and is generally believed to be between 40 and 60%.

The release of mMD, mAST and GLDH in liver disease has been studied by many investigators (Schmidt et al., 1967a; Gabrielli and Orfanos, 1968). Schmidt et al. (1967a) suggested that the varying behaviour of the mitochondrial enzymes GLDH, mAST and mMD might offer a precise indication of the extent and type of mitochondrial damage in liver disease. Equivalent studies, mainly on mAST, in myocardial infarction have not yielded conclusive results. Several investigators (Fleisher et al., 1960; Boyde and Latner, 1962) reported the occurrence of mAST in the serum of patients with myocardial infarction. Later, Massarat and Lang (1965) studied 10 patients with the disease and found that mAST was detectable on the first day of the infarct; they concluded that the release of mAST was mainly a phenomenon characteristic of the first 24 hours. Boyde (1968a) reported that mAST returned to normal at a slower rate than the total serum activity and suggested that the

enzyme was released gradually from the cell. He further observed (1968b) that mAST activity was found to be raised even when the total serum activity had returned to normal, and thus regarded mAST as a more sensitive index of myocardial infarction than total AST. Murros et al. (1973) examined the serum mAST in 24 patients with recent myocardial infarcts. They also suggested that mAST was gradually released from the damaged myocardium but, in contrast to the report of Massarat and Lang (1965), they were able to detect mAST in serum for longer than the first day after the infarct.

In recent years considerable effort has been spent on attempts at improving the specificity of enzyme tests as well as on searching for an accurate index for the severity of the infarct. Although none of the mitochondrial enzymes mentioned here is "heart-specific", it is possible that by virtue of their subcellular location they could indicate more accurately than cytoplasmic enzymes the extent of cell necrosis and hence infarct size.

### 3-2      Clinical material and methods of assay.

#### 3-2-1      Clinical material.

The clinical study was carried out in the period September 1973 - July 1974 on patients in the Royal Infirmary of Edinburgh. The patients studied consisted

of 3 groups:

Group 1 Patients with suspected myocardial infarction  
admitted to the Coronary Care Unit (C.C.U.). There were altogether 142 patients, selected on a random basis (one in three) on admission to the unit. Three patients died before sufficient data had been obtained and hence were discarded from the study. The remaining 139 patients (109 males; 30 females) were divided into 4 classes according to ECG and clinical findings, the enzyme activity results not being considered in making the classification:-  
Class 1 "Proven" myocardial infarction. .

The clinical symptoms and ECG findings of 68 patients fulfilled the criteria for "very probable myocardial infarction" (WHO 1959).

Class 2 Possible myocardial infarction.

The ECG of 17 patients showed less distinct diagnostic abnormalities, but the patients were nevertheless considered by the doctors concerned to have had myocardial infarcts on the basis of the clinical findings.

Class 3 Myocardial ischaemia.

39 patients had probable cardiac pain accompanied by ECG findings which were either normal or showed only transient changes.

Class 4 Non-infarction.

There were 15 patients in whom the diagnosis of ischaemic heart disease was considered unlikely.

Group 2 Patients without myocardial infarction, but with disorders known to cause raised serum CK activity.

- Class 1 170 patients who had undergone non-cardiac surgical operations varying from cystoscopy to major abdominal surgery.
- Class 2 36 patients who had undergone cardiac bypass operations mainly for valve replacement.
- Class 3 9 patients on haemodialysis for acute renal failure.
- Class 4 4 patients with Grades III and IV coma due to barbiturate poisoning.
- Class 5 A "miscellaneous" group comprising 1 patient with hypothermia and 1 patient with the "Munchausen Syndrome".

Group 3 Control group.

132 apparently healthy male blood donors, aged between 30 - 65 years, served as a control group for determining the reference values for the activity of some enzymes in serum.

Only the 68 patients with proven myocardial infarction (Group 1 : Class 1) were involved in the study of the mitochondrial and cytoplasmic enzymes to be described in this section. The other groups were studied in the investigation of the MM and MB isoenzymes of CK described in the next section.

3-2-2 Enzyme analysis.

For Group 1 patients, blood samples were collected on admission to the Coronary Care Unit and subsequently at

intervals of 6 hours for the first 24 hours and thereafter at about 12 hourly intervals until discharged from the C.C.U. - usually between 60 - 72 hours after admission.

For Groups 2 and 3 patients, with the exception of the bypass patients where 2-3 serial samples were obtained from each patient, only single samples of blood were taken.

The blood samples were allowed to clot and serum was separated within 2 hours of sample collection. Enzyme tests were performed as soon as possible, mostly within 24 hours of receiving the sample; all tests were performed within 3 days. The serum samples were stored at 4°C in the dark. All visibly haemolysed samples were rejected. The following enzyme assays were performed:-

1. Total creatine kinase activity by the method of Rosalki (1967) using reagents supplied by Boehringer Ltd. (Lewes, England).
2. Total aspartate aminotransferase, using a modification (Smith et al., 1970) of the method of Henry et al. (1960).
3. Alanine aminotransferase (ALT) using a modification (Smith et al., 1970) of the method of Henry et al. (1960). The purpose of the inclusion of this enzyme was to indicate possible liver involvement during the course of the disease.
4. NAD-dependent malate dehydrogenase. A method using malate as substrate was developed for this purpose (Section 2-3-3).
5. Urea-stable lactate dehydrogenase (USLD) using the method

of Brydon and Smith (1973).

6. Glutamate dehydrogenase using the method of Ellis and Goldberg (1972).

The evaluation of all these methods has already been described in Section 2.

Isoenzymes of AST, MD and CK were measured by methods to be described separately (Section 3-2-3 and Section 4-2).

### 3-2-3 Determination of the isoenzymes of AST and MD.

A simple chromatographic technique based on the method of Schmidt et al. (1967b) was used. It separates simultaneously the mitochondrial isoenzymes of both AST and MD from their cytoplasmic components.

#### Reagents.

1. Potassium phosphate buffer 8 mmol/l pH 7.0.
2. DEAE Sephadex A50 (Pharmacia, Uppsala, Sweden) pre-equilibrated in phosphate buffer.
3. Visking dialysis tubing (8/32).

#### Procedure.

The serum samples were dialysed overnight in Visking tubing against phosphate buffer. The pre-equilibrated Sephadex was packed into small columns (0.8 cm in diameter) to a height of about 10 cm. 1-2 ml of the dialysed serum was introduced into the column followed by elution with phosphate buffer. The eluates were collected in 5 fractions of about 3 ml each in a LKB UltroRac fraction collector



(No. 7000). The activity of mAST and mMD in each fraction was assayed using 0.7 ml of eluate in place of the usual 0.1 ml to increase sensitivity. The effect of dialysis on activity was corrected for by multiplying the total activity of all the fractions by the ratio:

Activity of the original sample/Activity of the dialysed sample.

In nearly all the samples studied, very little change of activity was observed on dialysis for either AST or MD, apart from a slight decrease attributable to the small increase in volume occurring as a result of dialysis. The activity of the cytoplasmic isoenzyme was obtained by subtracting the activity of the mitochondrial form from the total activity.

#### Assessment of the method.

For this purpose partially purified extracts of mAST, cAST, mMD and cMD were prepared from human heart. They were shown to be free from contamination with their other isoenzyme by electrophoresis in 7% polyacrylamide gel.

Various amounts of the four isoenzymes were added to inactive serum and then dialysed overnight. The dialysed samples were applied to the column which was then eluted first with the 8 mmol/l phosphate buffer followed by 0.2 mol/l potassium phosphate buffer pH 7.0 containing 0.2 mol/l NaCl, shown to be capable of eluting the cytoplasmic isoenzymes (Schmidt et al., 1967b).

The recovery of the mitochondrial and cytoplasmic isoenzymes is shown in Table 3-1. The precision of the

Tables 3-1

Recovery of Isoenzymes from Column Eluates

Enzymes	*Total Activity IU/l	Activity Recovered IU/l	% Recovery
mAST	2200	2400	110
	1190	1150	95
	780	750	96
	440	410	93
	120	134	110
	50	44	90
mMD	2000	1920	96
	1100	1200	110
	800	720	90
	460	420	91
	100	110	110
	40	38	94
	20	21	104
cAST	1140	1130	99
	840	760	91
	620	600	96
	440	400	92
	370	310	85
	350	320	90
	140	150	110
	90	88	98
	20	18	90
cMD	3900	3670	94
	2600	2700	104
	1600	1440	90
	600	640	107
	160	154	96
	80	70	88

\* For mitochondrial isoenzymes the recovery percentages referred to the first eluate (eluted with 8 mmol/l phosphate buffer pH 7.0); for cytoplasmic isoenzymes the recovery percentages referred to those of the second eluate (eluted by 0.2 mol/l phosphate buffer pH 7.0 + 0.2 mol/l NaCl) no activity being found in the first eluate in any of the samples.

method, calculated from duplicate analysis of samples, is listed in Table 3-2. Since almost all mAST was recovered in the first eluate (Table 3-1), it seems unlikely that the binding of mAST to  $\alpha_2$ -macroglobulin in serum (Boyde and Prime, 1968) was significant in the dialysed sera under the present experimental conditions.

### 3-3

#### Results.

#### 3-3-1

##### Determination of reference ranges.

The reference ranges for the activity of AST, MD, CK, USLD, ALT and GLDH in serum were determined from the 132 male blood donors, while those for mAST and mMD were obtained from a group of 68 people (55 male and 13 female). The distribution of these data was checked by plotting the cumulative frequency against enzyme activity on probability paper. It was found that AST, CK, USLD and ALT data had a log-normal distribution while MD, mAST, mMD and GLDH data fitted neither a normal nor a log-normal distribution (Fig. 3-1a and b). Their distributions were all skewed to the right. The reference ranges of these enzymes are listed in Table 3-3.

The reference range for GLDH was found to be higher than that reported by Ellis and Goldberg (1971), 0 - 4 IU/l, using the same assay method. As there are sex differences in the reference values of some enzymes, the following upper

Table 3-2

Repeatability of the Column Method

mAST IU/l				mMD IU/l			
144	140	44	45	60	66	20	18
124	104	36	39	50	52	16	15
106	107	26	26	30	33	10	12
99	103	20	27	30	32	2	5
90	94	18	19	25	30	0	1
86	80	14	15	20	24	0	1
58	61	2	3	-	-	-	-
S.D. = 4 IU/l (C.V. = 7.7%)				S.D. = 2 IU/l (C.V. = 10%)			

The standard deviation (S.D.) was calculated from the formula

$$\sqrt{\sum d^2 / (n-1)}$$

where d = difference between duplicates  
n = total number of observations

The coefficient of variation (C.V.) was obtained from S.D. / mean value.

Figure 3-1

Distribution of Serum Enzyme Activities in Healthy Individuals

a.

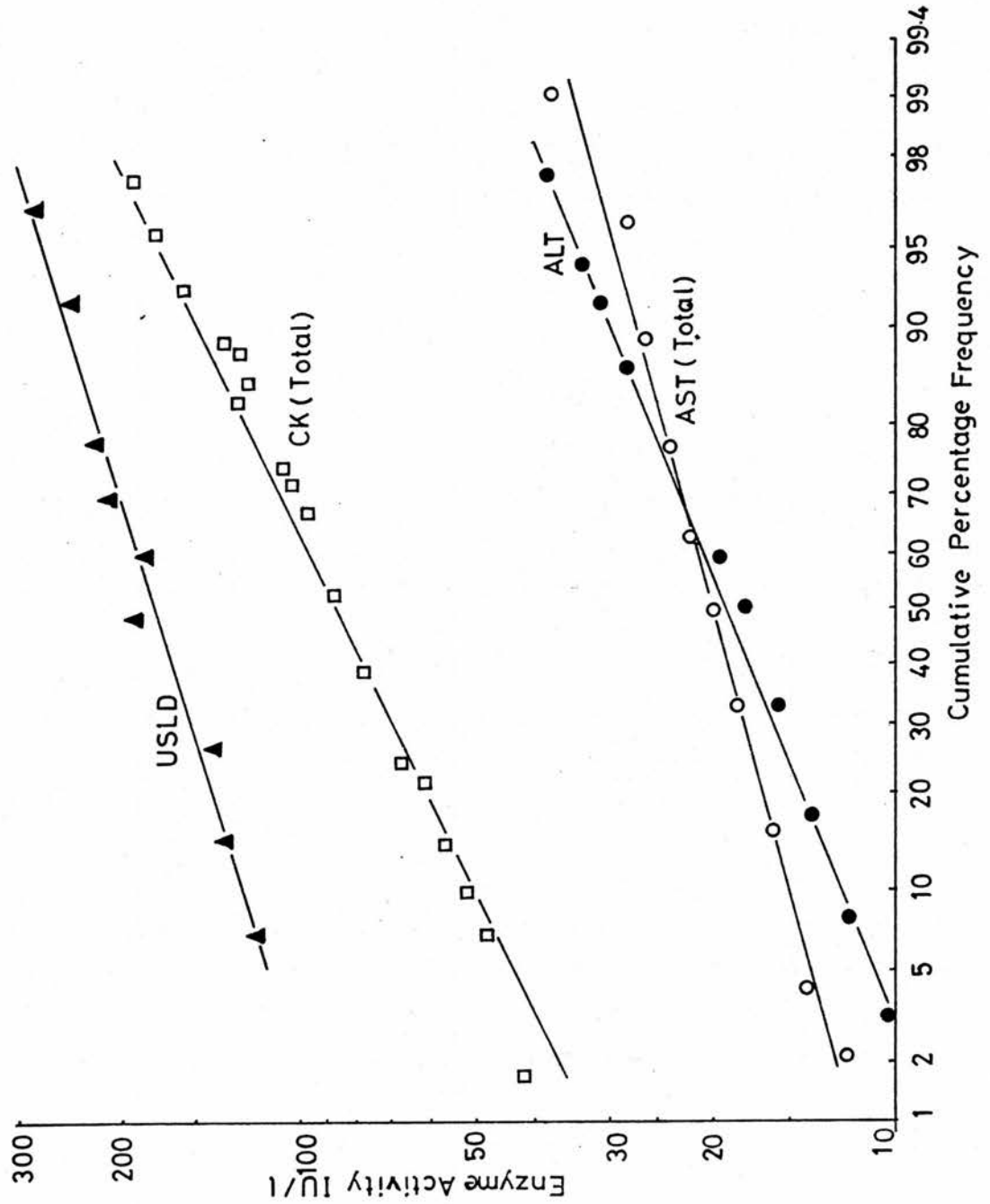
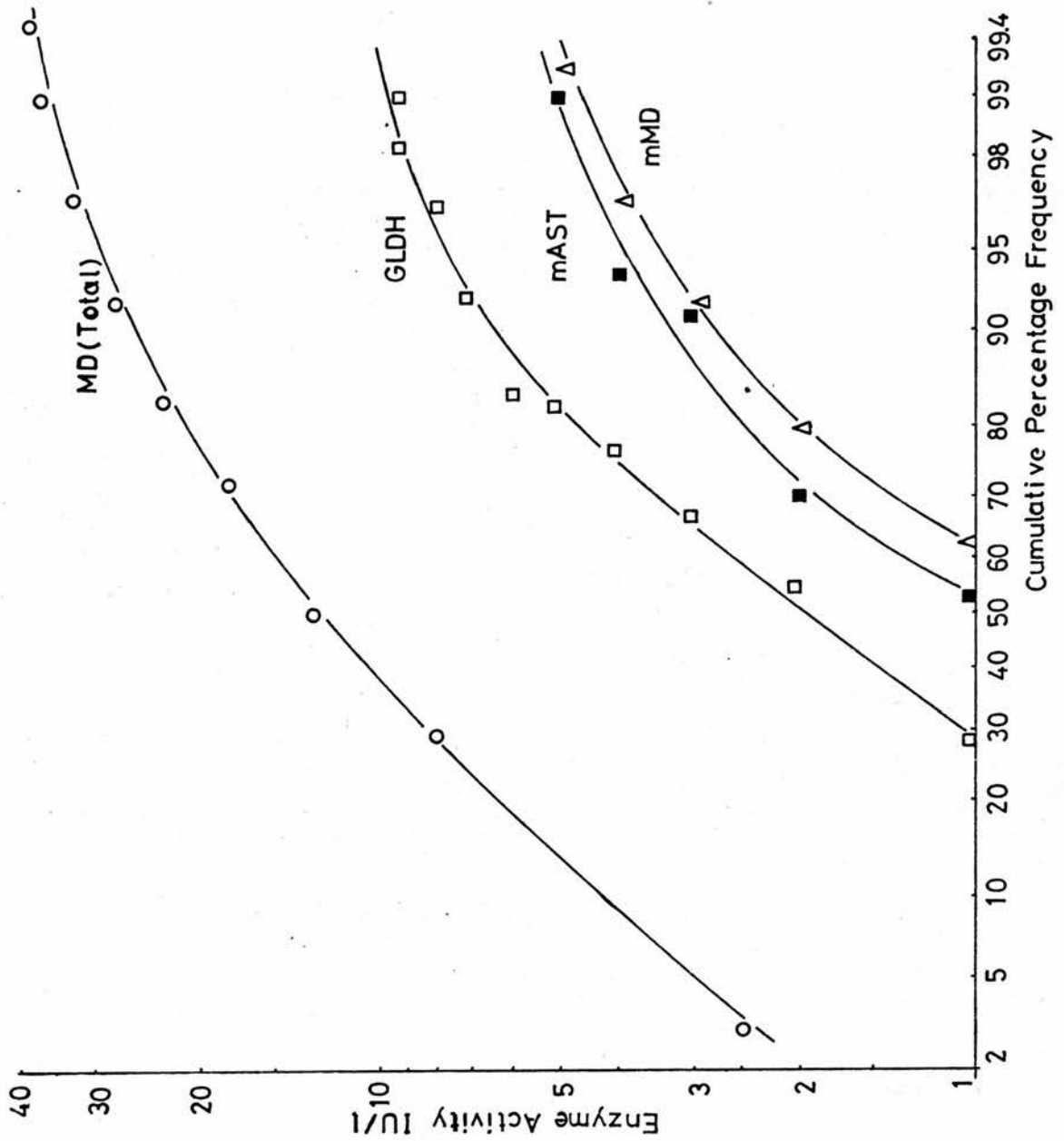


Figure 3-1

Distribution of Serum Enzyme Activities in Healthy Individuals

b.



A significant portion of the sample showed no detectable activity of mMD, mAST and GLDH; the assay methods (see text) used for these enzymes were not sensitive enough to detect activity between 0 - 1 IU/l.

Table 3-3

Reference Ranges for Serum Enzymes

Enzymes	No. of data	Mean IU/l	S.D. IU/l	Reference Range IU/l
CK	131	102	45	40 - 200
AST (Total)	99	20.2	4.9	10 - 30
USLD	98	203	49	100 - 300
MD(Total)	124	15	8	5 - 36
ALT	99	19.2	7.8	10 - 35
mAST	64	2	1.3	0 - 4
mMD	65	1.5	1.0	0 - 4
GLDH	58	4.0	2.3	0 - 7

reference limits for women were assumed based on previous studies in our laboratory as well as those in the literature (e.g. Goldberg and Winfield, 1974): AST and ALT 25 IU/1, CK 150 IU/1.

3-3-2      Release of cytoplasmic and mitochondrial enzymes into the circulation.

For each enzyme, the peak activity was the highest activity observed in the enzyme profile; the peak time was the time between the onset of symptoms and the time when the peak activity was observed.

Figure 3-2 shows the variation of serum enzyme activity with time in a typical patient; Table 3-4 shows the average peak time of mMD, mAST, cMD and cAST in serum after infarction. The peak times for each of the four enzymes all approximated to a normal distribution about their respective means. Paired Student's "t" test analysis shows that the two mitochondrial enzymes (mAST and mMD) reached peak values of activity in serum significantly later than their cytoplasmic isoenzymes. There is no significant difference between the average times for peak activity of mAST and mMD, whereas cMD reached its peak of activity significantly earlier than cAST.

The release of mAST, mMD, cMD, cAST and GLDH was followed for up to 130 hours after the onset of pain in some cases. The period was divided into 12 intervals, and the average activity of each of the serum enzymes and the percentage of



Figure 3-2

The Rise of Some Serum Enzymes in a Patient after Myocardial Infarction

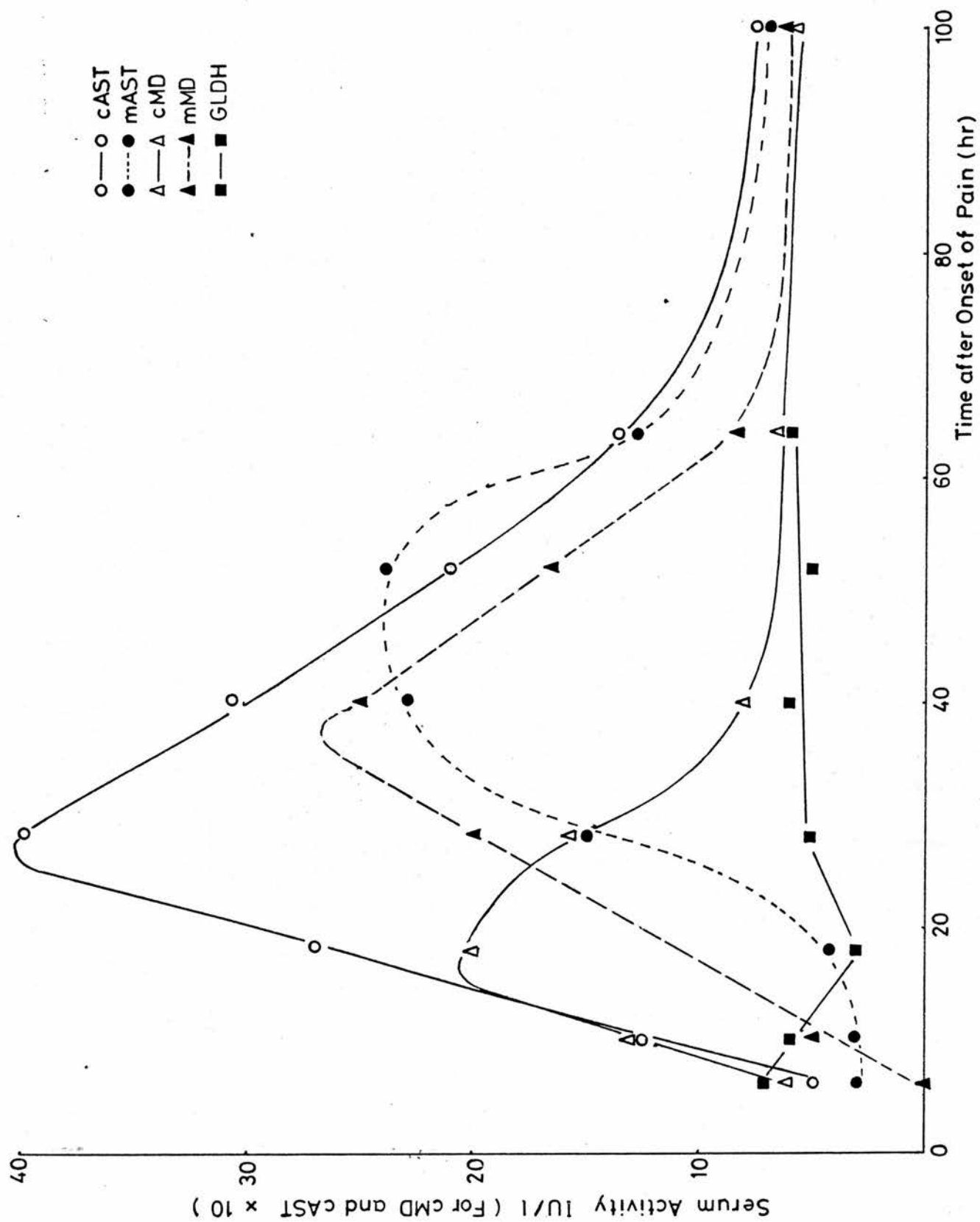


Table 3-4

Average Peak-time of the Isoenzymes of AST and MD  
in Serum after Myocardial Infarction

	mMD	mAST	cMD	cAST
No. of Patients	51	53	57	55
Average Peak-time (hr)	41.1	39.0	17.9	26.4
S.D. (hr)	14.0	12.0	7.5	8.3

Significance of the Difference between Average Peak-times  
of the Enzymes (by Paired Student's "t" test)

	mMD:mAST	mMD:cMD	mAST:cAST	cAST:cMD
Pairs of Data	50	51	52	55
"t"	1.4	9.9	8.1	9.7
Probability (P)	$0.1 > P > 0.05$	$P \ll 0.0005$	$P \ll 0.0005$	$P \ll 0.0005$

abnormal enzyme results in each interval was noted (Table 3-5). It can be seen that mAST and mMD became abnormal considerably later than their cytoplasmic isoenzymes.

The serum activity of GLDH did not rise to a distinct peak value in most patients studied. The average GLDH activity remained normal throughout the 130 hour period, although individual patients sometimes showed small rises.

### 3-3-3 Rate of clearance of enzymes from serum.

The rate of decay of enzyme activity was measured for each of the enzymes after the enzyme activity had reached its peak. Many of these clearance curves were found to show two components, an initial fast phase and a subsequent slow phase. The apparent overall half-life ( $T_{\frac{1}{2}}$ ) of the serum enzymes was obtained by tracing the enzyme profile to half-peak activity level for each patient; cMD was cleared fastest ( $T_{\frac{1}{2}} = 19.3$  hours) while mAST was the slowest ( $T_{\frac{1}{2}} = 36.6$  hours). The mean apparent  $T_{\frac{1}{2}}$  values were all significantly different from each other with the exception of mAST and mMD. The mitochondrial isoenzymes were cleared significantly more slowly than their cytoplasmic components (Table 3-6).

### 3-3-4 Abnormal peak activity of cytoplasmic and mitochondrial enzymes.

The peak activities of cAST, mAST, cMD, mMD and GLDH

Table 3-5

The Release of Cytoplasmic and Mitochondrial Enzymes in Patients  
with Proven Myocardial Infarction

Time after Pain (hr)	Mean Value				
	cAST IU/l	cMD IU/l	mAST IU/l	mMD IU/l	GLDH IU/l
0 - 6	32(63) *35%	42(63) 37%	1.8(55) 55%	1.4(55) 4%	4.2(54) 4%
6 - 12	99(59) 95%	102(59) 93%	3.0(58) 22%	2.4(59) 15%	4.0(48) 8%
12 - 18	180(49) 100%	147(49) 98%	5.7(51) 49%	3.7(51) 24%	3.0(40) 5%
18 - 24	204(48) 100%	125(48) 98%	8.2(47) 72%	4.9(47) 47%	3.8(36) 3%
24 - 30	228(32) 100%	106(32) 91%	14.7(29) 86%	9.2(30) 67%	4.3(27) 7%
30 - 36	212(31) 100%	82(31) 77%	16.5(31) 90%	10.3(30) 67%	4.6(25) 16%
36 - 42	193(31) 94%	63(31) 77%	15.4(23) 96%	10.0(24) 79%	4.1(22) 14%
42 - 48	171(26) 100%	52(27) 67%	16.0(24) 92%	11.8(24) 75%	6.1(15) 27%
48 - 54	131(18) 100%	54(17) 65%	14.2(22)100%	9.5(22) 68%	4.4(21) 10%
54 - 66	112(29) 100%	39(29) 55%	14.5(38) 89%	9.2(33) 67%	5.7(35) 20%
66 - 80	74(34) 97%	30(33) 24%	12.7(30) 80%	9.7(30) 73%	6.4(25) 32%
80 - 130	47(28) 79%	30(28) 18%	5.7(27) 49%	4.0(27) 35%	6.7(17) 22%

\* = percentages of abnormal results

() = number of data

Table 3-6

Rates of Decay of the Released Enzymes (Apparent  $T_{\frac{1}{2}}$  Values)

	mAST	cAST	mMD	cMD
No. of Patients	31	49	28	49
Mean $T_{\frac{1}{2}}$ (hr)	36.6	30.5	32.8	19.3
S.D.	12.4	7.4	16.7	6.8

Significance of the Difference between the Mean  $T_{\frac{1}{2}}$  Values of the Enzymes (using the Student's paired "t" test)

	mAST:mMD	mAST:cAST	mMD:cMD	cAST:cMD
No. of Pairs of Data	26	28	25	46
"t"	1.16	3.27	3.69	10.16
Probability (P)	$0.15 > P > 0.10$	$0.0025 > P > 0.0005$	$0.0025 > P > 0.0005$	$P \ll 0.0005$

were compared with the respective values for the upper limit of the reference range. Cytoplasmic AST, cMD and mAST had raised peak activities in all patients studied, 93% of the patients had abnormal peak mMD activities but only about 40% had abnormal peak GLDH activity. The highest rises in activity observed in the present study were cAST - 19 times, mAST - 14 times, cMD - 13 times, mMD - 11 times and GLDH - 9.5 times their respective upper reference values.

The peak values of cAST, mAST, cMD, mMD and total CK were then compared with each other. A regression analysis was done on each comparison (Table 3-7). It was found that the activities of the mitochondrial enzymes correlated well with each other ( $r = 0.9$ ), and so did the cytoplasmic enzymes ( $r = 0.88$ ) (Fig. 3-3a and b). However, the correlation between cytoplasmic and mitochondrial enzymes was poorer ( $r$  ranging from 0.59 to 0.75).

### 3-3-5 Correlation of cytoplasmic and mitochondrial enzymes with degrees of heart failure.

The patients with myocardial infarcts, in whom sufficient data were available, were placed into four clinical grades depending on the presence and extent of heart failure: none, mild, moderate and severe. Table 3-8 shows that in general there is a relationship between the severity of heart failure and the magnitude of the peak value of activity, for all four enzymes. The mitochondrial enzymes

Table 3-7

Correlations between the Peak Values of Serum Enzymes

	mAST:mMD	mAST:cAST	mMD:cMD	cAST:cMD	mMD:CK	mAST:CK
Sample Size	60 pairs	61 pairs	62 pairs	66 pairs	58 pairs	57 pairs
r	0.9	0.75	0.59	0.88	0.70	0.72

r = coefficient of correlation

Figure 3-3

- a. Correlation between the Peak Activities of Mitochondrial MD and AST in Serum after Myocardial Infarction

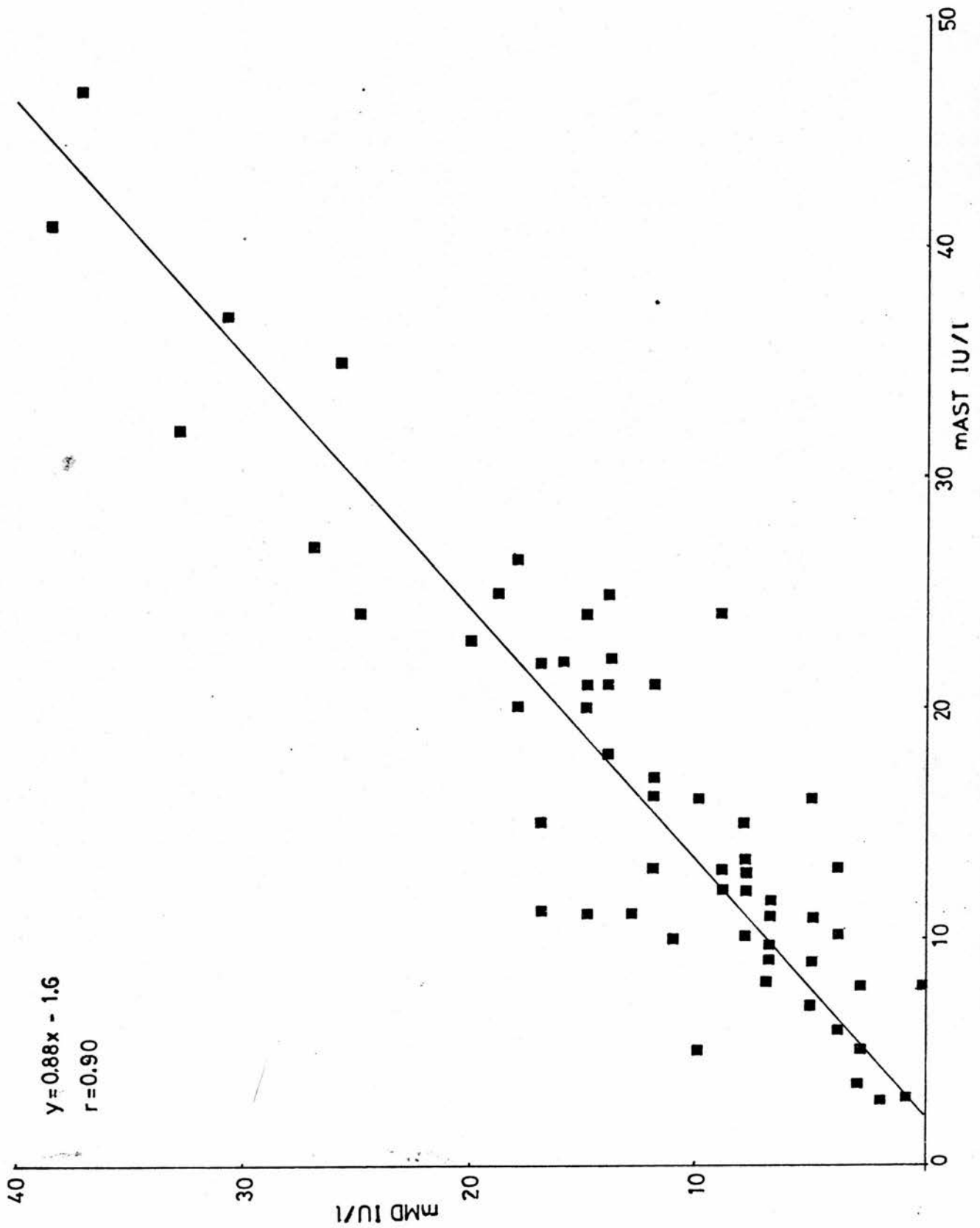




Figure 3-3

- b. Correlation between the Peak Activities of Cytoplasmic MD and AST in Serum after Myocardial Infarction

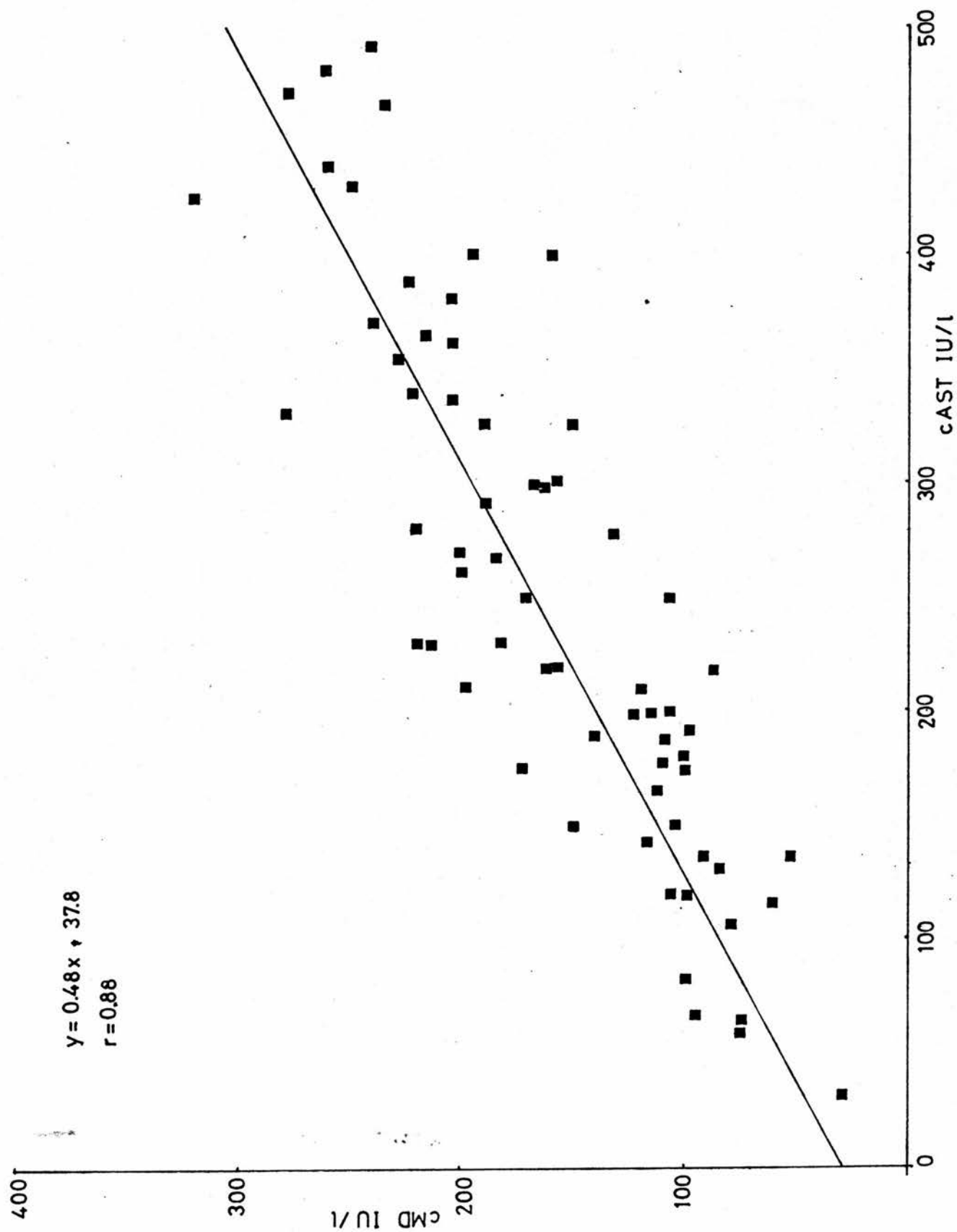


Table 3-8

Correlation between the Release of Enzymes and Different  
Degrees of Post-infarct Heart Failure

	None	Mild	Moderate	Severe
No. of Patients	27	10	11	8
Mean Activity IU/l(S.D.):				
mAST	15(8.2)	20(11)	21(12)	34(13)
cAST	245(108)	262(130)	267(120)	370(98)
mMD	11(6.4)	14(6.0)	17(8.5)	23(10)
cMD	158(70)	165(56)	173(96)	213(32)
*mAST	-	0.15 > P > 0.10	0.10 > P > 0.05	P < 0.0005
*cAST	-	P = 0.35	0.30 > P > 0.25	0.01 > P > 0.005
*mMD	-	0.10 > P > 0.005	0.05 > P > 0.025	P < 0.0005
*cMD	-	P = 0.40	0.30 > P > 0.25	0.025 > P > 0.0125

\* = Significance of the difference between mean activities of various groups with respect to "None" ; values of P were obtained by the Student's "t" test.

seemed to show a better correlation than the cytoplasmic, since patients with "severe" heart failure showed average mitochondrial AST and MD peak activities that were 2.1 times those from patients with no heart failure; the corresponding figure for cytoplasmic AST and MD was 1.4 times.

3-4

#### Discussion.

Mitochondrial enzymes and cell necrosis. The first manifestation of cell injury due to ischaemia (or any other cause) is cell swelling as a result of disturbance in the sodium pump and ATP production in the cell (Ginn et al., 1968). The permeability of the cell membrane changes and it is conceivable that at this stage certain cytoplasmic enzymes could be released from the cell, especially those with small molecular weight. So far the change in the cell is completely reversible, with no apparent functional damage. If, however, the injury continues, derangement of the intracellular organelles occurs. At this point the mitochondria begin to swell, assuming bizarre shapes and sizes, followed by rupturing of their membranes. It is presumably during this phase that the mitochondrial enzymes are released. With this irreversible damage to the cell's respiratory apparatus, death of the cell almost certainly ensues.

The present serum enzyme findings seem to be compatible with this sequence of events. The release of cAST and cMD was significantly earlier than the corresponding release of mAST and mMD (Table 3-4). This is in agreement with the

hypothesis that the cytoplasmic enzymes begin to "leak" during the initial reversible phase of cell injury. This hypothesis seems to be further supported by the fact that the release of the cytoplasmic enzymes correlated poorly with the release of the mitochondrial ones (Table 3-7), a result to be expected if only cytoplasmic enzymes were released in the early stage of injury. However, more conclusive evidence is necessary for validation of this hypothesis.

It is likely that the majority of the mitochondrial enzymes - mAST, mMD and GLDH, leak out of the cell during the rupturing stage rather than the swelling stage of the mitochondria, since these enzymes (being in the inner membrane matrix) have to pass through both the inner and outer membranes of the organelle. If this is true, these mitochondrial enzymes will offer a much better indication of cell death (and hence infarct size) than the cytoplasmic enzymes in cases where there is a large area of affected tissue surrounding a small necrotic centre. The present semi-quantitative analysis (Table 3-8) on heart failure seems to support this view. A rigorous quantitative validation of this suggestion in the present study would have been difficult as the mitochondrial enzymes (mAST and mMD) took a long time (more than 100 hours after the infarct) to return to normal, by which time patients had already been discharged from the Coronary Unit. Therefore, peak activity values had to be used in place of the total area under the graph of enzyme activity against time which would have been a more accurate

index of the total amount of enzyme released. Moreover, there were very few deaths in the present study, and the physical assessment of the infarct size at autopsy was therefore not feasible. Instead, a clinical phenomenon (heart failure) related to the infarct size had to be used, but the degree of heart failure is also influenced by many factors other than infarct size.

Another major disadvantage in using mAST or mMD to assess cell death in myocardial infarction is the fact that these enzymes, though specific to mitochondria, are not specific to myocardium; they may derive from other organs, especially the liver. Failure to recognise this may lead to gross overestimation of the degree of myocardial damage.

GLDH was slightly raised in many of the patients studied and normal in the rest. This is perhaps related to its high molecular weight (1 000 000) and its presence in only small quantities in the myocardium. However, a very high value (350 IU/l) was observed in 1 patient who also had high values of mAST (140 IU/l) and mMD (56 IU/l). This patient was later shown not to have had a myocardial infarct but to be suffering from liver disease.

Clearance of the enzymes from serum. The measurement of  $T_{\frac{1}{2}}$  values for the clearance rates of enzymes from serum in patients can only be approximate. The apparent  $T_{\frac{1}{2}}$  value obtained is a composite of the mode of release of the enzyme, mixing between body fluids and the multiphasic clearance of the enzyme. The data from the present study show that the

apparent half-lives of mAST and mMD in serum after myocardial infarction are similar (36.6 and 32.8 hours respectively) and tend to be longer than the corresponding half-lives for cAST and cMD (30.5 and 19.3 hours respectively) (Table 3-6). Animal experiments, in which known amounts of enzyme were injected, suggested a much shorter half-life for mAST (0.5 - 1.0 hours) (Fleisher et al., 1963) and for cAST (8 - 12 hours) (Fleisher et al., 1963; Schmidt et al., 1967a), whereas those for mMD (about 28 hours) and cMD (about 14 hours) (Schmidt et al., 1967a) were of the same order as those found for the human enzymes in this study.

If the data from these animal experiments are applicable to humans, then the most likely explanation for the long apparent half-lives of mAST and cAST in human serum after myocardial infarction would be that the enzymes continued to be released for several days after the episode. Furthermore, it would be necessary to postulate that the pattern of release of mMD and cMD was dissimilar from that of the AST isoenzymes, with most of the mMD and cMD being released as single discharges after infarction. The report of Murros et al. (1973) on the release of mAST agrees with this hypothesis. However, it seems unlikely that mMD and mAST should have different manners of release since they are located in similar sites in the mitochondrion. It is thus doubtful whether the extrapolation of results from animal experiments to humans is entirely justified, although mAST in different species has been found to be very similar (Gehring et al., 1975), and injection experiments on a single

patient (Dawson, et al., 1969) gave  $T_{\frac{1}{2}}$  values of similar magnitude (for cMD and total AST) as in the animal experiments described above. Further experiments appear to be needed before valid conclusions can be drawn from these studies.

The mitochondrial and cytoplasmic enzymes as indicators of myocardial infarction. In all the 68 patients with proven myocardial infarction cAST, cMD and mAST were raised, while 93% had raised mMD and about 40% had raised GLDH. The maximum peak heights (relative to the upper limit of the reference range) were higher for the cytoplasmic than the mitochondrial enzymes. Also they began to show their maximum frequency of abnormal results (about 12 hours after the onset of pain) earlier than the mitochondrial (24 - 30 hours after the onset of pain) (Table 3-5). In terms of sensitivity and the early detection of the infarction, it seems that the mitochondrial enzymes are less suitable than the cytoplasmic. Although mAST had a longer apparent half-life than cAST, the finding of Boyde (1968b) that mAST remained raised when total AST activity had returned to normal was not evident in the present study.

Of the two cytoplasmic enzymes, cAST is superior to cMD as a diagnostic aid in myocardial infarction. The released cAST stays in the blood longer and rises to a higher value. Comparison of the two mitochondrial enzymes shows that mAST is better than mMD in supporting the diagnosis of infarction, since mAST rises to a higher value and is detectable in a higher percentage of patients with the disease.



Patterns of release of mitochondrial enzymes in myocardial infarction compared with those in liver disease.

Mitochondrial AST, mMD and GLDH are present in many tissues and are known to be released in a number of pathological conditions other than myocardial infarction e.g. liver disease. It is interesting to compare the release of these enzymes in myocardial infarction with their release in liver disease, using data from the reports of Schmidt et al. (1967a), and Gabrieli and Orfanos (1968):-

1. The ratio mMD/cMD in serum is higher in hepatitis (mMD being 25 - 35% of the total MD activity) than in myocardial infarction (mMD less than 20% of the total enzyme activity).
2. The ratio mAST/cAST in serum is higher in myocardial infarction (mAST about 14% of the total enzyme activity) than in liver disease (mAST about 8% of the total).
3. Proportionately more GLDH is released in liver disease than in myocardial infarction, although the comparison here is not strictly valid as two different methods of assay were used. The few GLDH assays on patients with liver disease in the present study, however, agree with this view.
4. The rise in activity of mAST, mMD and GLDH in serum is slower in liver disease than in myocardial infarction, probably due to the different nature of cellular damage involved.



Conclusion. In a group of 68 patients with proven myocardial infarction, the mitochondrial isoenzymes of AST and MD were found to be released into the blood later and to have a longer apparent half-life than the corresponding cytoplasmic isoenzymes. Their peak activities did not correlate well with those of the cytoplasmic enzymes. This is presumably due to the additional release of the cytoplasmic enzymes during the reversible swelling of the cell prior to permanent damage. In terms of sensitivity and early detection of the disease, the cytoplasmic isoenzymes are superior to the mitochondrial. The only diagnostic value of the mitochondrial isoenzymes studied seems to be their greater specificity for indicating cell necrosis, an assessment of which may be useful for estimating prognosis.

SECTION 4

THE RELEASE OF CREATINE KINASE ISOENZYMES  
IN MYOCARDIAL INFARCTION

Introduction.

This Section is particularly concerned with a comparative study of the release of the MM and MB isoenzymes of creatine kinase in patients with suspected myocardial infarction. It compares the results of these studies with other enzymes (AST, USLD and ALT) that are frequently investigated in patients with this condition.

Creatine kinase was first shown to be of diagnostic value in muscular dystrophy by Ebashi et al. (1959). In the following year, Dreyfus et al. (1960a) detected the enzyme in normal serum and further observed (1960b) that CK activity may be elevated up to 10-25 times the upper limit of the "normal range" in patients with myocardial infarction. Since then the diagnostic value of CK in myocardial infarction has been confirmed by many investigators (Hughes, 1962; Konttinen and Holonen, 1963; Smith, 1964; Duma and Siegel, 1965).

There have been many studies on the specificity of this enzyme, at one time thought to be specific to muscular damage, but further work revealed that serum CK activity was raised in a number of diseases with no apparent muscular involvement. Creatine kinase has been found in the thyroid gland (Graig and Smith, 1966) and the lung (Perkoff, 1968), and it is released into the blood in pneumonia and pulmonary embolism as well as in hypothyroidism (Graig and Ross, 1963). Serum CK activity is also occasionally raised in patients with cerebral infarcts (Hess et al., 1964), cerebrovascular accidents (Kalbag et al., 1966) and psychotic disorders

(Meltzer, 1968). Even when muscular injury is present, the release of CK has been found to be by no means confined to myocardial infarction and muscular dystrophy. Plasma enzyme activity may be raised in normal individuals after strenuous exercise (Griffiths, 1966). It may also be elevated following accidental and surgical trauma (Eshchar and Zimmerman, 1967), administration of drugs by muscular injection (Meltzer et al., 1970) and various forms of cardiac disease - myocardial trauma, acute rheumatic carditis, paroxysmal tachycardia and pericarditis (King and Zapf, 1972).

The use of isoenzymes in improving the diagnostic specificity of CK for myocardial infarction has only been studied relatively recently. The minor component, the MB isoenzyme, is mainly present in appreciable quantity (about 30%) in human heart tissue (Eppenberger et al., 1970; Dawson and Fine, 1967). The potential value of this relatively heart-specific isoenzyme has not yet been fully evaluated although a preliminary report about its release in myocardial infarction was published nine years ago by Van der Veen and Willebrands (1966), who stated that the MB isoenzyme was present in serum in muscular dystrophy. More recently, a number of reports have suggested that the MB isoenzyme might be a better test for myocardial infarction than total serum CK activity (Konttinen and Somer, 1972; Roe et al., 1972; Smith, 1972).

The following study attempts to assess the diagnostic potential of the MB isoenzyme in patients with myocardial infarction and to compare its pattern of release, its clearance from the blood and its diagnostic specificity

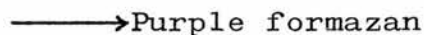
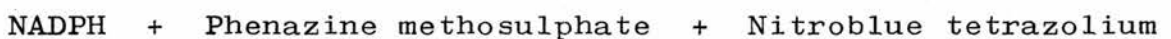
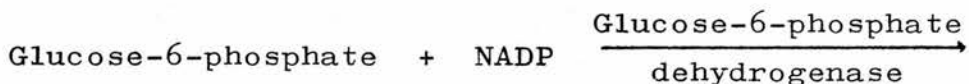
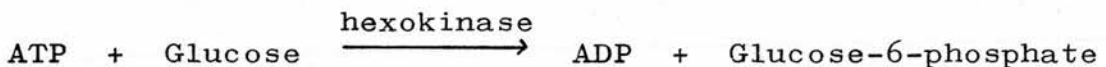
with those of the more abundant MM isoenzyme.

#### 4-2

#### Materials and methods.

The classification of patients and the collection of serum samples have been described in Section 3-2. The assay of total CK activity and its isoenzymes was performed in all three groups of patients.

The MM and MB isoenzymes were assayed after separation by electrophoresis on polyacrylamide gel, using a technique based on the method of Smith (1972). Some modifications were made to optimise the method for quantitative measurements. The isoenzyme activity was demonstrated on the gel by staining via two coupled enzyme reactions:



#### Reagents and apparatus.

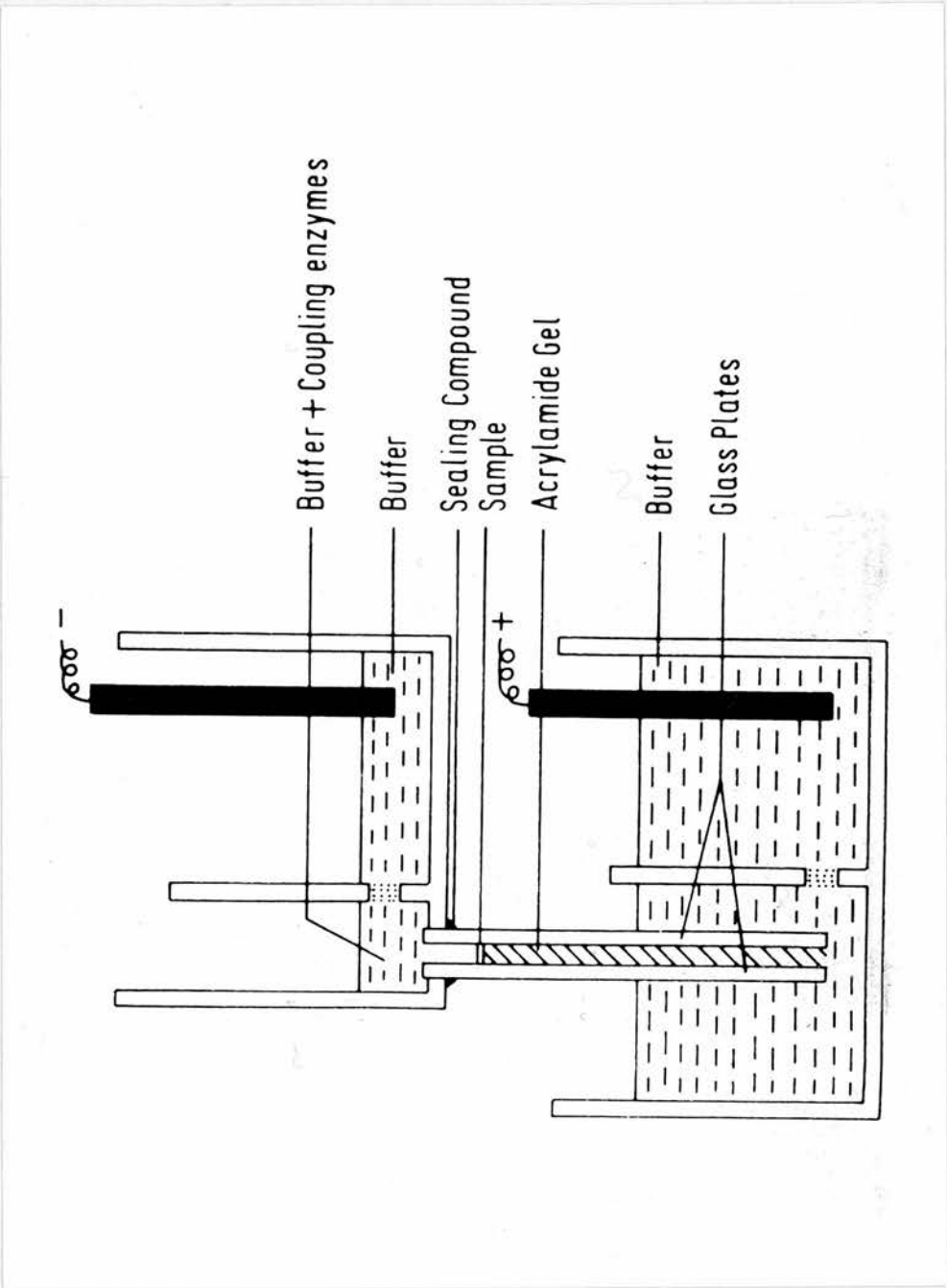
1. 7% polyacrylamide gel slab (containing 5% bisacrylamide) was prepared fresh in a glass plate compartment (20 x 8 x 0.2 cm).

2. Buffer in electrophoretic tanks and in gel: 0.08 mol/l tris-EDTA-borate pH 8.4.
3. Electrode tanks. Both anode (25 x 10 x 7 cm) and cathode (25 x 7 x 7 cm) tanks were made of perspex with a single platinum wire running along the length of the tank. The upper cathode tank was divided along its length into two compartments: the larger (5 cm wide) contained the platinum electrode and was separated from the smaller (1.5 cm wide) by a perspex sheet containing, near its lower border, three holes (0.7 cm in diameter) plugged with cotton wool. A slit (20 x 0.8 cm) for the insertion of the polyacrylamide electrophoresis cell was cut in the bottom of the smaller compartment of the cell (Fig. 4-1). The cell was then temporarily sealed into position by a flexible sealing compound (Bostik Ltd., Leicester, England).
4. Sample compartment spacers. Soft rubber tubing 2.5 mm in outer diameter and 1 cm long.
5. Staining solution:

<u>Reagents</u>	<u>Final concentration</u>
Creatine phosphate	35 mmol/l
ADP	2 mmol/l
AMP	20 mmol/l
Magnesium acetate	10 mmol/l
Glucose	20 mmol/l
NADP	1 mmol/l
Nitro-blue tetrazolium (NBT)	30 mg in 15 ml
Phenazine methosulphate (PMS)	1 mg in 15 ml

Figure 4-1

Design of the Apparatus for Electrophoresis of Creatine Kinase  
Isoenzymes in 7% Polyacrylamide Gel Slab



All were dissolved in 0.1 mol/l tris-acetate buffer pH 7.0 in a total volume of 15 ml. The solution of reagents without NBT and PMS ( which were added fresh) was stored at  $-20^{\circ}\text{C}$ .

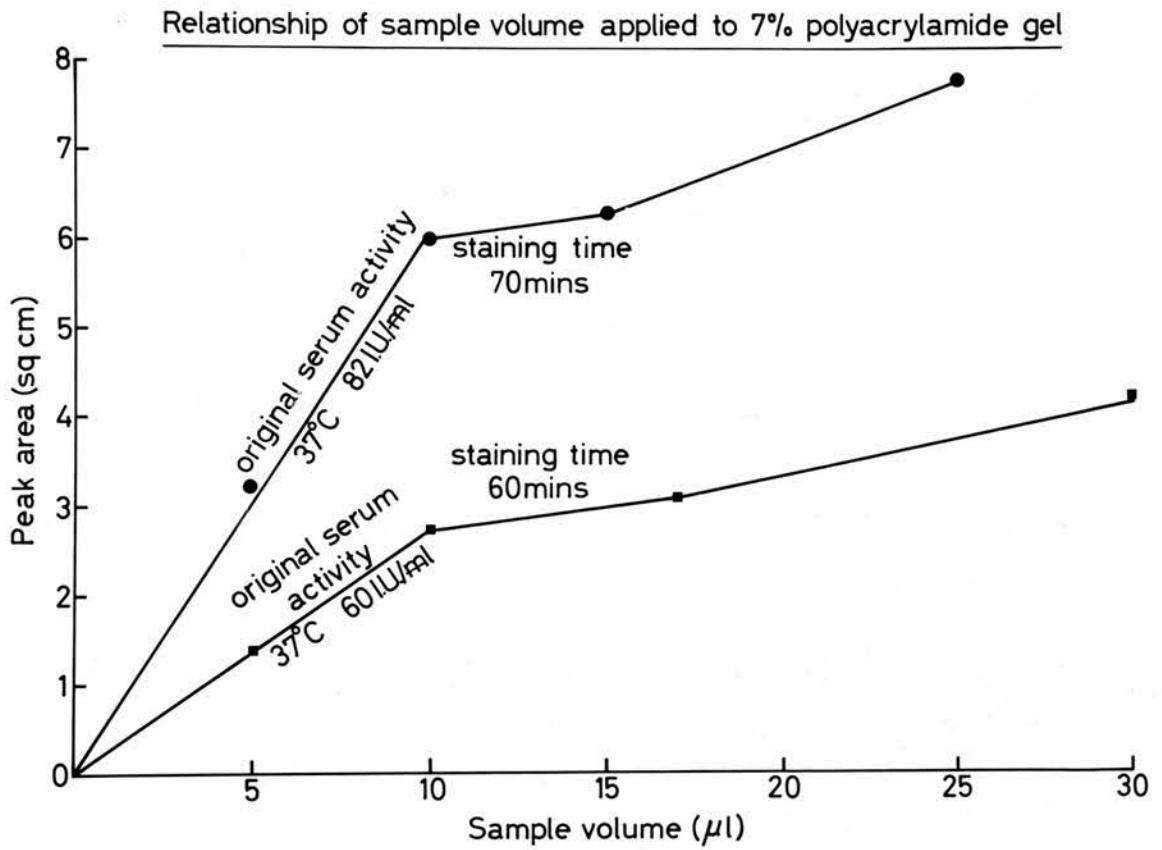
#### Procedure.

Temporary sample compartments were made on the gel by placing the sample spacers 0.5 cm apart. The electrophoresis cell was inserted into the bottom slit of the upper cathode compartment as described. The lower anode tank was filled with the tris-EDTA-borate buffer and the electrophoresis cell, with attached cathode, placed in the tank. 100 ml of the same buffer, to which 50IU hexokinase and 10 IU glucose-6-phosphate dehydrogenase had been added, was placed in the smaller cathode compartment, and the larger compartment filled to the same depth with buffer containing no added enzyme.

The coupling enzymes were introduced into the gel by a 45 - 60 min preliminary run at 400 volts. The current was switched off and 0.5 ml 30% bovine albumin and a further 50 IU hexokinase and 10 IU glucose-6-phosphate dehydrogenase added to a smaller cathode compartment. A 10  $\mu\text{l}$  sample (Fig. 4-2), diluted if necessary in 40% sucrose to an activity of 200 IU/l, was applied to the gel and 5  $\mu\text{l}$  of bromophenol blue added to one of the compartments to act as a marker. Electrophoresis was stopped when the complex of bromophenol blue with albumin had migrated 4-5 cm. The gel was taken out and immersed in 1 mmol/l  $\beta$ -mercaptoethanol for 20 min, followed by 0.1 mmol/l tris acetate pH 7.0 for the same



Figure 4-2



The total peak area was obtained by staining the gel with a substrate solution for the activity of creatine kinase isoenzymes followed by scanning with a densitometer as described in the text. The serum sample volume applied was found to be linear with the total peak area so obtained within the range 0 - 10  $\mu$ l. Hence 10  $\mu$ l was chosen as the standard sample volume.

period. It was then incubated at 37°C with 15 ml staining solution for 70 min (Fig. 4-3). After staining, the gel was rinsed with 5% acetic acid and immediately scanned on a Vitatron TLD 100 "flying spot" densitometer (Fisons Scientific Apparatus, Loughborough, England) using the instrument in the "transmission mode" with a 560 nm filter in position. Immediate scanning was desirable to avoid adverse effects due to slight fading of the bands (Fig. 4-4).

The percentage of MB isoenzyme in the sample was calculated by measuring the areas under the MM and MB isoenzyme peaks. The MB isoenzyme activity was then calculated from the formula:-

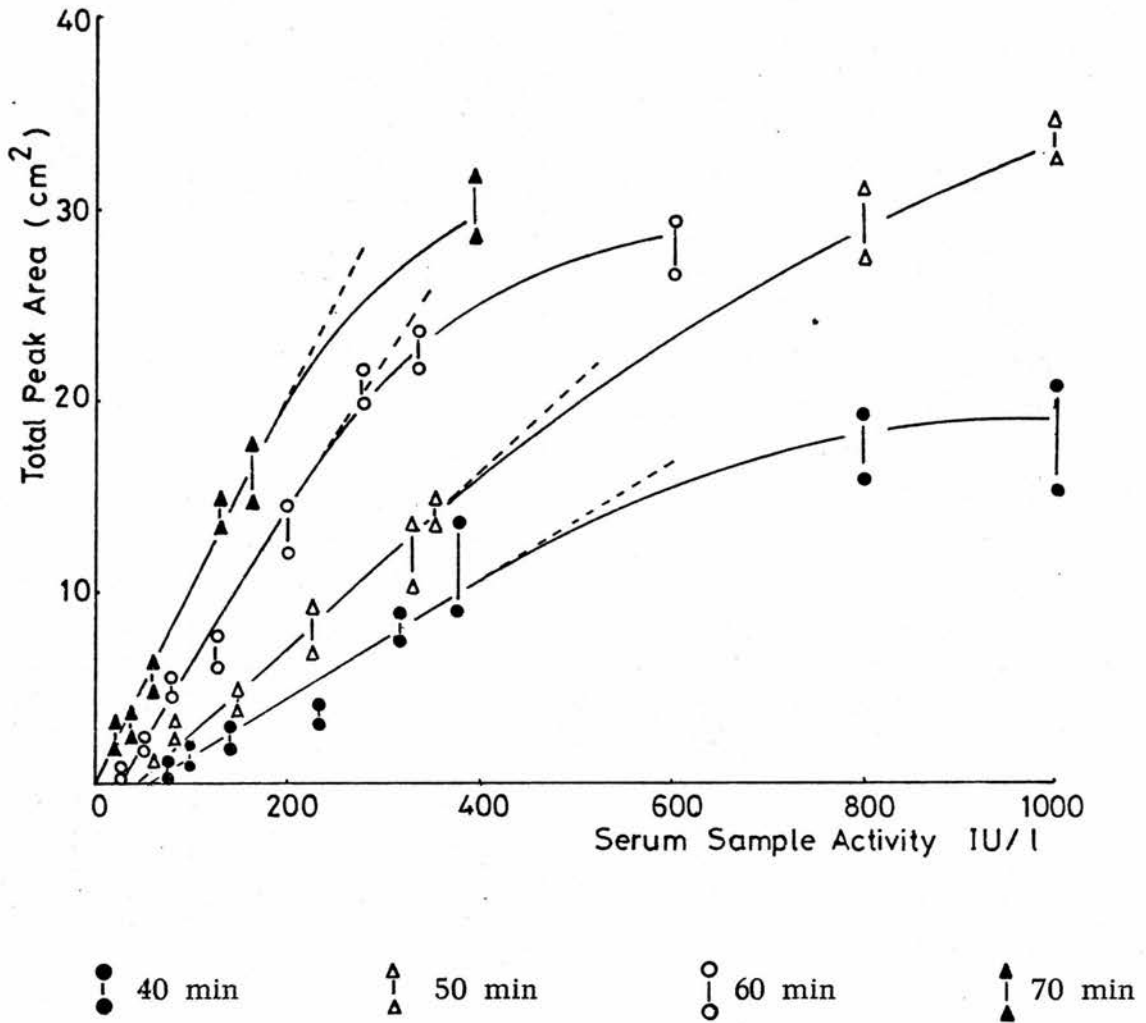
$$\frac{(\text{Area under MB peak} / \text{Area under MM + MB peaks}) \times \text{total CK activity}}{\text{total CK activity}}$$

The MM isoenzyme activity was obtained by subtracting the MB isoenzyme activity from the total. For each gel electrophoresis, a control strip was cut off the gel and stained for activity of the coupling enzymes, hexokinase and glucose-6-phosphate dehydrogenase, to ensure their even distribution through the gel (Fig. 4-5).

The assessment and validation of this method is considered separately, in Section 6.

Figure 4-3

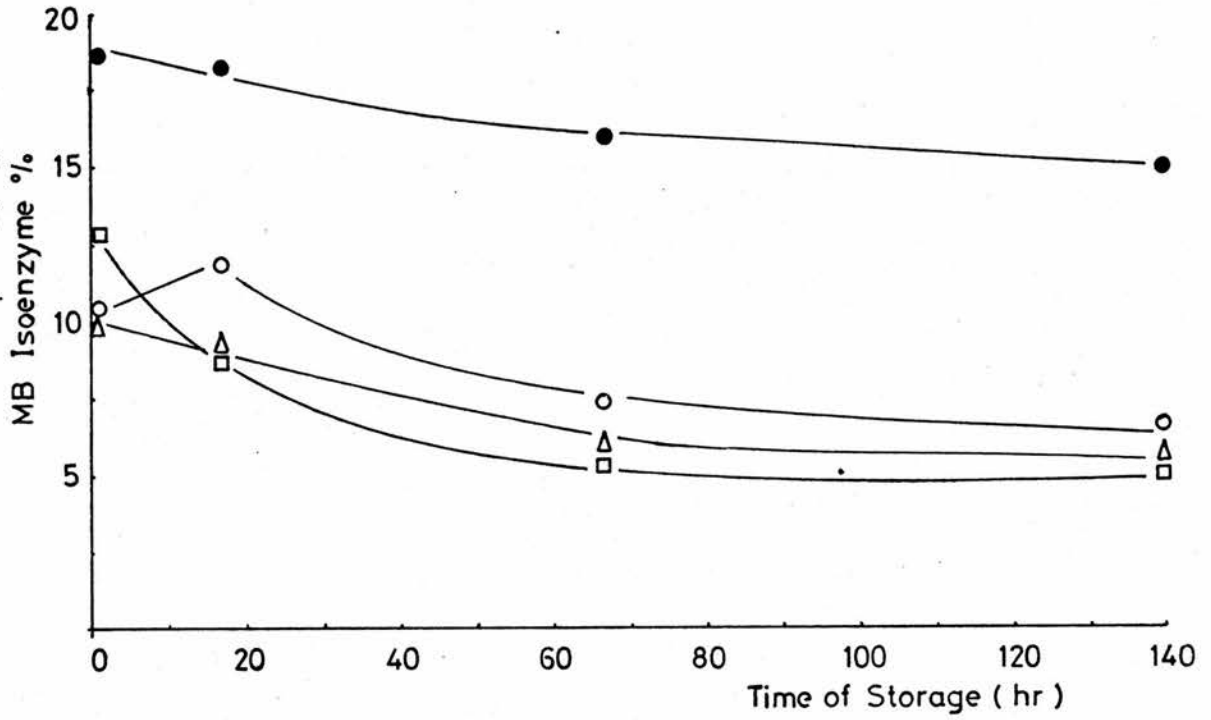
Relationship between Stained Peak Area and Serum Sample Activity  
with Different Incubation Period at 37°C



From graph it can be seen that the sensitivity of the polyacrylamide gel method increases with increasing incubation time. In the range of incubation periods investigated, the 70 min incubation was the most satisfactory. With this incubation period the maximum serum sample activity should not exceed 200 IU/l if the enzyme activity is to be linearly related to peak area. All active serum samples in the present study were therefore diluted accordingly.

Figure 4-4

Effect of Storage in 5% Acetic Acid on the Intensity of the  
Stained CK Bands in 7% Polyacrylamide Gel



Total CK activity:

□ 90 IU/l;

Δ 230 IU/l;

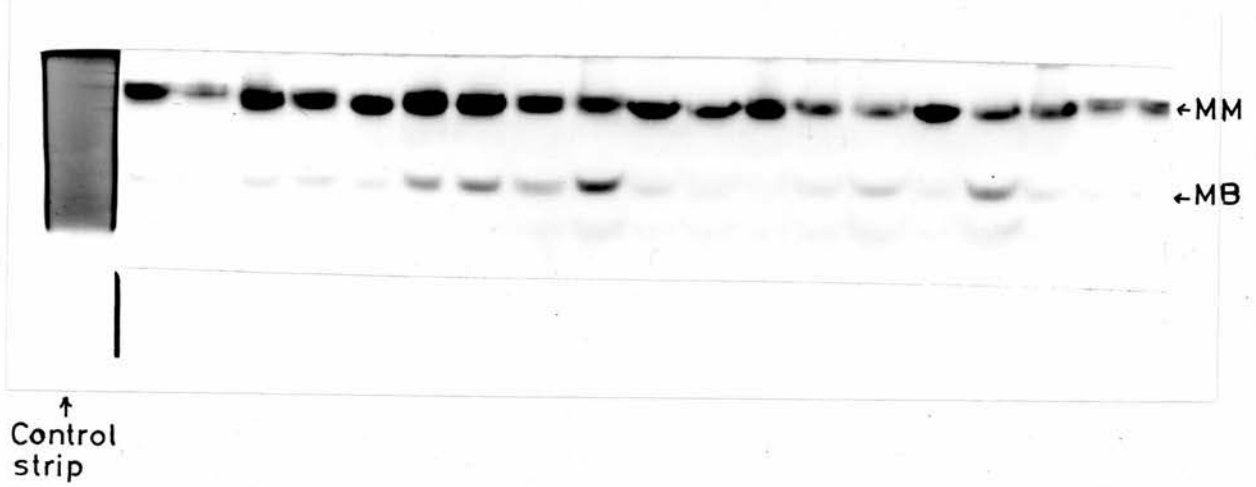
○ 145 IU/l;

● 344 IU/l

In the range of serum CK activities studied, a slight fading of the bands (with a resultant decrease in the MB% of the total peak area) was evident in the first 65 hours of storage.

Figure 4-5

Serum CK Isoenzyme bands after Electrophoresis in 7%  
Polyacrylamide Gel



The control strip was stained for the coupling enzyme activities in a substrate solution containing ATP (1 mmol/l), Magnesium acetate (10 mmol/l), Glucose (20 mmol/l), NADP (1 mmol/l), NBT (2 g/l) and PMS (0.7 g/l) in a total volume of 10 ml.

#### 4-3-1 Reference ranges for the MB and MM isoenzymes.

No MB isoenzyme band was detected on the gel in any of the 132 blood donors. Although the electrophoretic method is able to demonstrate activity as low as 5IU/1 in diluted tissue extracts, it cannot detect with certainty any activity below 10 IU/1 in serum due to lack of sensitivity and precision. Therefore, 10 IU/1 was assumed to be the upper limit of the reference range of the MB isoenzyme. This could well be an overestimate in view of the following data published recently by other workers. Henry et al. (1975), using batch adsorption on glass beads, reported the reference serum MB isoenzyme activity to be 1.6 IU/1 (S.D. = 0.28); Nealon and Henderson (1975), who applied concentrated plasma to an ion-exchange column, found a reference range of 1.6 - 7.0 U/1; Yasmineh and Hanson (1975) obtained a value of 0.8 U/1 (S.D. = 0.5) using stepwise elution chromatography.

The reference range of the MM isoenzyme was assumed to be that of the total CK as the other component, the MB isoenzyme, occurs only in very small quantities. This means that the upper limit for the reference activity of the MM isoenzyme is 150 IU/1 for females and 200 IU/1 for males (Section 3-3-1).

#### 4-3-2 The pattern of release of the MM and MB isoenzymes after myocardial infarction.

Only those enzyme data obtained from the 68 patients with proven myocardial infarction (Group 1 - Class 1) were

used for the analysis. In all those patients from whom sufficient data were obtained, the magnitude of the peak activity of the isoenzymes, the peak time and the time needed for activity to fall to half the peak value (apparent  $T_{1/2}$ ) were noted. The enzyme profile of Class 1 patients is shown in Fig. 4-6.

The maximum serum activity of the MB and MM isoenzymes, AST and USLD is listed in Table 4-1. The mean rise of MB isoenzyme activity (22.2 times the upper reference value) was much greater than that of the MM isoenzyme, total CK, total AST or USLD.

The incidence of abnormal serum levels of the MM and MB isoenzymes, total CK, AST and USLD at timed intervals after the onset of infarction is listed in Table 4-2 and shown in Fig. 4-7. The MB isoenzyme had the highest frequency of abnormal results in the period up to 18 hours. In the period 19-36 hours all the enzymes had similar frequencies of abnormal results. From the 37th hour onwards the frequency of abnormal serum MB isoenzyme activity began to fall rapidly. As might be expected the frequency of abnormal results for the MM isoenzyme activity was similar to that of total CK activity.

The individual values for the peak activity of the MB isoenzyme did not correlate well with the corresponding values for the MM isoenzyme of CK or with total AST activities from the same patient. On the other hand, the peak activities of the MM isoenzyme of CK and total AST correlated well with each other (Table 4-3). The proportion of the MB isoenzyme

Figure 4-6

Mean Serum Enzyme Activities in Group 1 - Class 1 Patients  
at Varying Intervals after Myocardial Infarction

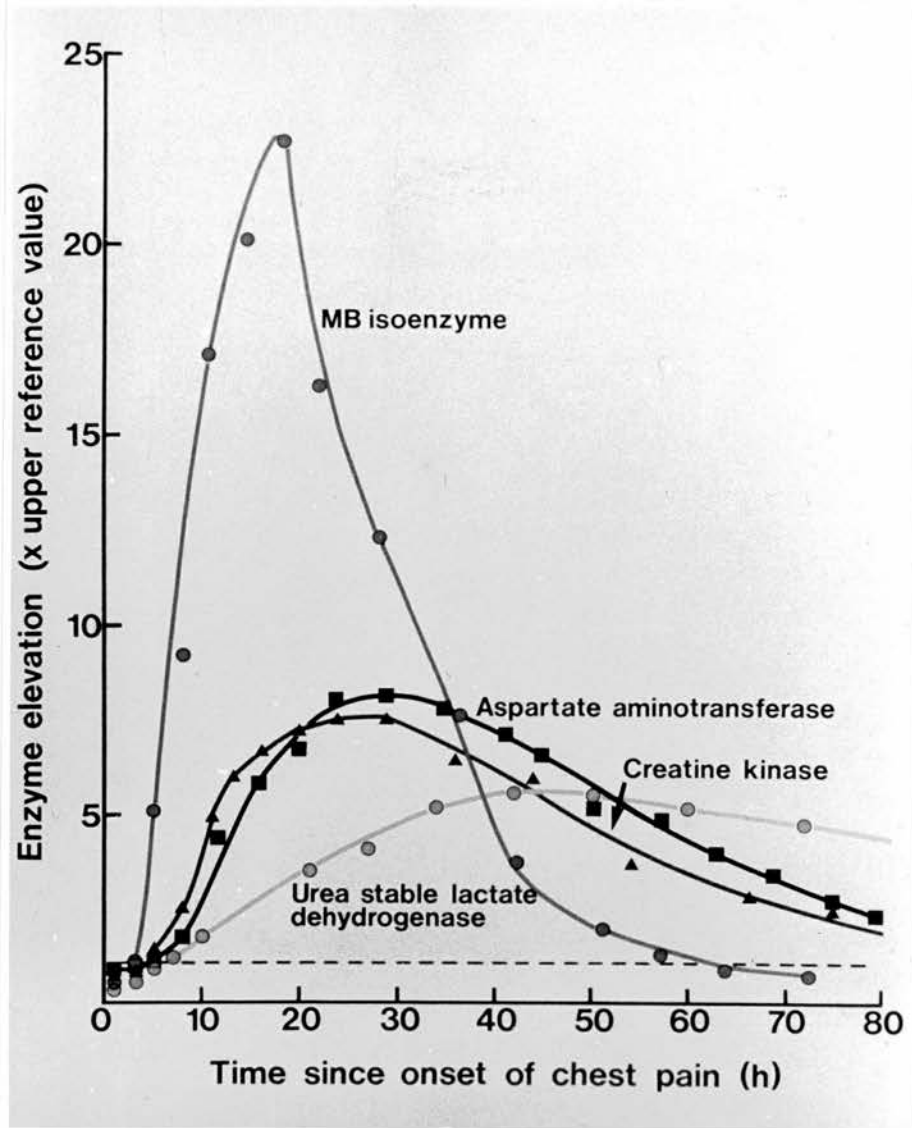




Table 4-1

Maximum Enzyme Activity Results in Patients  
with Myocardial Infarction

Enzyme	Mean IU/l	Range IU/l	x Upper Reference Mean	Range
Total CK	1720	300-3200	8.6	1.5-16
MM Isoenzyme	1650	240-3000	8.2	1.2-15
MB Isoenzyme	220	28-700	22.2	2.8-70
Total AST	250	39-600	8.3	1.3-20
USLD	1710	360-420	5.7	1.2-14

Figure 4-7

Incidence of Some Abnormal Serum Enzyme Levels after Myocardial Infarction in Group 1 Patients

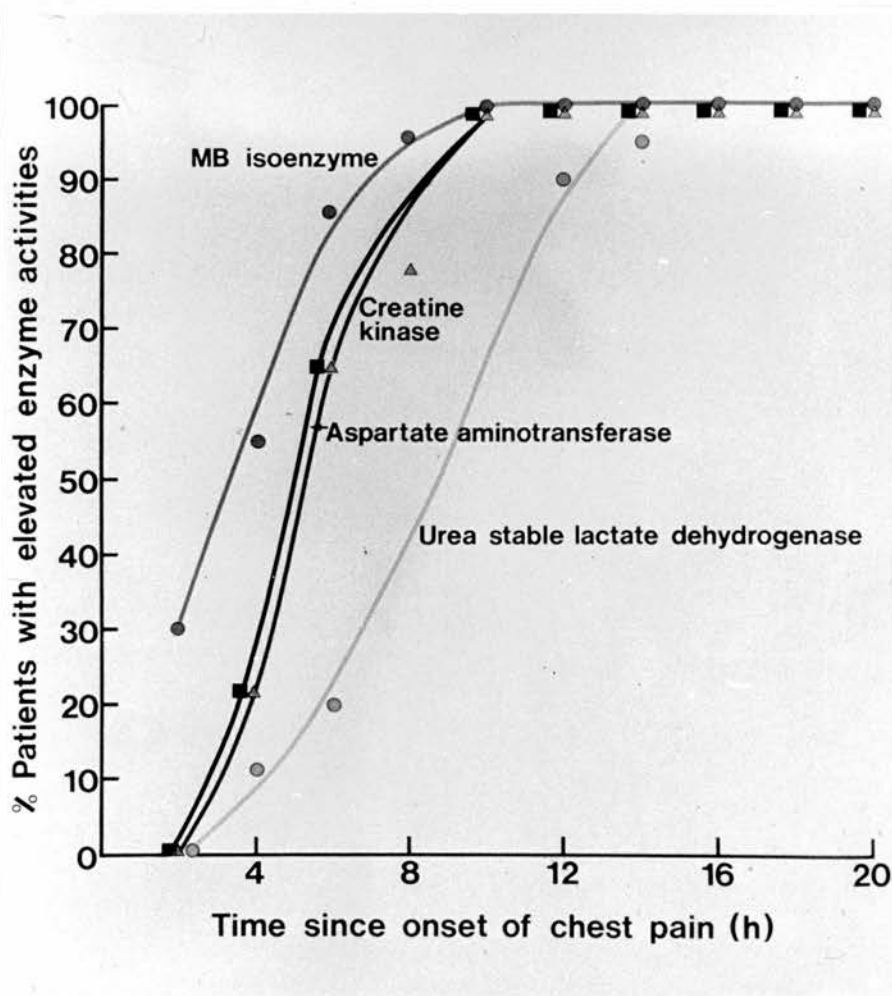


Table 4-2

Incidence of Abnormal Serum Enzyme Activities at Varying  
Intervals since Onset of Symptoms of Myocardial Infarction

Intervals since Infarction (hr)	% Abnormal Results				
	CK (MM)	CK (MB)	CK(Total)	AST	USLD
0 - 6	25	58	28	32	18
7 - 12	90	98	93	95	78
13 - 18	98	100	98	98	96
19 - 24	94	96	94	94	96
25 - 36	97	94	97	97	98
37 - 48	98	66	98	98	100
49 - 72	83	41	85	95	100

Table 4-3

Correlation between Peak Activities of Serum Enzymes

	MB:AST	MB:MM	AST:MM
r	0.60	0.45	0.90
No. of Pairs of Data	58	58	57

r = coefficient of correlation

to total CK activity in the specimen showing peak activity of the MB isoenzyme was also very variable, ranging from 4% - 30% (Table 4-4). A review of the clinical symptoms of the patients showed that the percentage of the MB isoenzyme contributing to the total activity of CK did not seem to be related to the age or sex of the patient, to the site of the infarct nor to any history of previous infarcts or angina.

It was found that the MM and MB isoenzymes of CK reached peak values at different times and were cleared from the circulation at different rates. In 58 patients with proven myocardial infarction in whom sufficient data were available, the average time for reaching peak activity of the MM isoenzyme in serum was 24 hours after onset of clinical symptoms; in contrast, the corresponding figure for the MB isoenzyme was 17 hours. Similarly, the MB isoenzyme was cleared much more rapidly from the circulation than the MM isoenzyme, having an apparent  $T_{1/2}$  value of 12.8 hours compared with 28.5 hours for the MM isoenzyme. (Table 4-5) Like those of the AST and MD isoenzymes, most decay curves of both MM and MB isoenzymes of CK consisted of two components, an initial rapid and a subsequent slow phase.

As in Section 3, the patients were grouped according to their different degrees of post-infarct heart failure (none, mild, moderate and severe) with their respective serum MM and MB isoenzyme peak activities (Table 4-6). There was a significant rise in average activity of the MM isoenzyme between the "none" and the "severe" groups.

Table 4-4

Variability of MB Isoenzyme Percentage at MB Isoenzyme Peak

% MB Isoenzyme at MB Isoenzyme Peak	No. of Patients	Mean Total CK at MB Isoenzyme Peak (IU/l)
Not Detectable	0	-
0 - 4	1	-
5 - 9	12	1020
10 - 14	9	1080
15 - 19	17	940
20 - 24	13	827
25 - 29	6	1051
30 +	10	1024

Table 4-5

Peak-time and Half-life of the MM and MB Isoenzymes in Serum

	MM Isoenzyme		MB Isoenzyme	
	Mean	S.D.	Mean	S.D.
Peak-time (hr)	24 (58)	7.3	17 (58)	4.5
Apparent Half-life (hr)	28.5 (57)	9.5	12.8 (57)	4.3

( ) = number of data from which peak-times and half-lives were calculated.

The significance of the difference between MM and MB isoenzymes in i) mean peak-time and ii) mean apparent half-life has been found to be  $P < 0.0005$  in each case by the Student's paired "t" test.

Table 4-6

Correlation between Peak Activity Values of CK Isoenzymes with  
Different Grades of Post-infarct Heart Failure in Patients with  
Proven Myocardial Infarction

	None	Mild	Moderate	Severe
No. of Patients	29	9	11	8
Mean Peak Activity IU/l (S.D.)				
MM Isoenzyme	1420 (779)	1850 (670)	1760 (736)	2260 (627)
MB Isoenzyme	196 (85)	300(106)	320 (118)	290 (90)
*MM Isoenzyme	-	0.10 > P > 0.05	0.15 > P > 0.10	0.05 > P > 0.025
*MB Isoenzyme	-	0.05 > P > 0.025	P ≐ 0.01	P ≐ 0.025

\* = significance of difference between mean values with respect to "None"  
as calculated from the Student's "t" test.

However, no significant rise was detected in the "mild" and "moderate" groups for this isoenzyme. For the MB isoenzyme there was a distinct rise in activity from patients without heart failure to those with heart failure. The average activities in the "mild", "moderate" and "severe" groups, however, were not significantly different from each other.

4-3-3 The diagnostic specificity of the MB isoenzyme of creatine kinase in myocardial infarction.

The results of the studies of the pattern of release of the MB isoenzyme have shown that it becomes abnormal earlier, reaches a peak value earlier and returns to normal earlier than the MM isoenzyme of CK or any of the other enzymes studied. It also rises to higher maximum values in terms of its upper reference limit than any of the other enzymes investigated (Table 4-1). These properties of the MB isoenzyme have prompted further study of its diagnostic specificity for myocardial infarction.

Group 1 Patients with suspected myocardial infarction admitted to the Coronary Care Unit (see Section 3-2-1).

Class 1: Patients with "proven" myocardial infarction.

In all 68 patients the serum activities of the MB and MM isoenzymes of CK, AST and USLD were raised. The details of the rise have been described above.

Class 2: Patients with possible myocardial infarction.

Twelve of the 17 patients had raised activities of the MB isoenzyme as well as raised activities of the MM isoenzyme, AST and USLD. The overall pattern of elevation in these patients both in terms of their relative activities and the time course of elevation was highly suggestive of myocardial infarction. Consequently, there was little doubt that these patients had, indeed, had myocardial infarcts.

Two other patients had raised values for the MB isoenzyme only, all other enzymes activities being within their reference ranges. One of these patients developed an unequivocal ECG pattern of infarction 4 days after admission, the other also developed highly suggestive sequential ECG changes. It was very probable that these 2 patients had also had small myocardial infarcts.

A further 2 patients in whom no enzyme rose above its reference range had had two previous infarcts rendering their ECG findings difficult to interpret at the time of admission. Although diagnosed as probably having had infarcts, more detailed evaluation of the clinical data by several cardiologists led to the conclusion that it was impossible to give a firm opinion as to whether recent infarction had occurred.

The remaining patient, whose only raised serum enzymes were the aminotransferases (peak values - AST: 180 IU/l; ALT: 196 IU/l), had had angina for a week. Although her ECG pattern was suggestive of myocardial infarction, it was difficult to date the occurrence of the infarct. The



infarct may therefore have occurred some days before admission; this would explain the absence of raised MB isoenzyme activity as well as other enzymes.

Class 3: Patients with myocardial ischaemia.

Ten of the 39 patients in this group showed elevations characteristic of myocardial infarction with all the enzymes studied. In another 19 patients the serum enzymes were normal. In the remaining 10 patients some enzymes were elevated: 4 had a rise of only AST, 1 had raised AST and USLD, 3 had a raised activity of the MM isoenzyme of CK, 1 had raised activity of the MB isoenzyme of CK, and 1 had a rise in all enzymes except the MB isoenzyme. From their clinical symptoms and ECG findings these changes were attributed to causes other than myocardial infarction (Table 4-7).

Class 4: Patients having non-cardiac disorders. Out of these 15 patients, 10 had normal results for serum enzyme activity measurements. In the remaining 5, one or more of the enzyme results were abnormal. They were grouped together with others in Table 4-7 as being caused by disorders other than myocardial infarction.

In summary, in this group of 139 patients admitted to the Coronary Care Unit, 92 were thought to have had myocardial infarction (namely 68 from Class 1, 14 from Class 2 and 10 from Class 3) of whom 90 showed elevation in the activities in serum of all the cytoplasmic enzymes that were studied. The remaining 2 only had an increase in the MB isoenzyme of CK.

In the 47 patients not thought to have had a myocardial

Table 4-7

Abnormal Enzyme Results in Patients with Chest Pain due  
to Causes Other Than Myocardial Infarction

Diagnosis	Enzyme Results IU/l			
	MB Isoenzyme	CK	AST	USLD
MB Isoenzyme Mainly Affected				
Paroxysmal atrial fibrillation	45	260	50	420
Pericarditis	40	N	N	N
Unexplained Chest Pain	28	N	N	N
CK Mainly Affected				
Unexplained (myositis ?)	N	664	68	320
Following Dental Extraction	N	460	N	N
Unexplained (3 cases)	N	300 (av)	N	N
Stokes Adams Attack	N	300	N	350
AST Mainly Affected				
Unexplained*	N	N	420	N
Heart Failure*	N	N	230	340
Alcohol Excess*	N	N	100	N
Heart Failure*	N	N	90	630
Unexplained*	N	N	69	N
Unexplained*	N	N	46	N
Unexplained (5 cases)	N	N	60 (av)	N
USLD Mainly Affected				
Haemolysis	N	490	80	3700
Heart Failure	N	N	34	520
Unexplained (2 cases)	N	N	N	500 (av)

\* These patients all showed increased serum alanine aminotransferase activity.

N = within the reference range; av = average value.

infarction, 17 had at least one of the enzymes with raised activity in serum. This group is important in the study of the diagnostic specificity of the serum enzymes concerned. In these 17 patients, only 3 had raised MB isoenzyme activity that was not attributable to myocardial infarction. Two of these patients can have their findings explained by other myocardial disorders - tachycardia and pericarditis. The remaining patient, a man of 32 with a history of alcoholism, presented with chest pain which clinically did not seem to be of cardiac type; the MB isoenzyme (28 IU/1) was the only abnormal enzyme finding and the ECG was normal.

Six patients had raised total CK activity in serum that was not caused by myocardial infarction. One of these patients (260 IU/1) had a marked tachycardia, and had an elevation of the MB isoenzyme (45 IU/1). Of the remaining 5 patients, one (460 IU/1) had recently had a dental extraction and another (300 IU/1) had had a Stokes-Adams attack, a condition previously shown to be associated with a rise in CK activity in serum (Smith, 1967), but no reason could be found for the other three. In all these 5 cases the MB isoenzyme was undetectable, showing its better diagnostic specificity than the MM isoenzyme or the total CK.

AST seems to be the least specific of all the enzymes studied here. There were altogether 11 patients with elevations not due to myocardial infarction. If AST results are considered in combination with ALT results, however, the diagnostic specificity improves appreciably: six of these 11 patients had a concurrent rise of ALT suggesting the

presence of hepatocellular damage, and two of the 6 had been drinking heavily prior to admission. The remaining 5 patients (peak values of AST: 76, 47, 68, 69, 42 IU/l) belonged to the unexplained category.

There were 4 patients with raised USLD activities not attributable to myocardial infarction. One was probably at least partly due to haemolysis (3700 IU/l), one due to heart failure (520 IU/l) and 2 were unexplained (340, 630 IU/l) (Table 4-7).

Group 2 Patients without myocardial infarction (Table 4-8).

Class 1: Postoperative cardiac bypass patients. An average of three serum samples were taken from the patients at about 12 hourly intervals within the first 2 days after operation. Most of these patients had received valve-replacements with periods on bypass usually between 2 and 4 hours. All the 36 patients had raised serum total CK activity, with values ranging from 240-2300 IU/l. Of these, 24 had no detectable MB isoenzyme bands. The MB isoenzyme percentages in the rest were: 6 with less than 5%, 5 with between 5 and 10% and 1 with 15%. The MB isoenzyme percentages here seem to be smaller than those observed in myocardial infarction.

Class 2: Non-cardiac postoperative patients. A single random serum sample was taken from patients in the period after operation up to 48 hours. These patients had undergone a wide range of surgical operations, ranging from cystoscopy to oesophago-gastrectomy. Forty six of the 170 patients had raised total CK activity (200-2500 IU/l). In none of them was the MB isoenzyme detected. Since only

Table 4-8

MB Isoenzyme Findings in Patients with Diagnosis  
Other Than Myocardial Infarction

Category	No. of Patients	No. with Raised CK Activity	Range of CK Activities IU/l	MB Isoenzymes in Patients with Raised CK IU/l			
				Not Detectable	10-49	50-99	Over 100
Cardiac Bypass	36	36	240-2300	24	6	5	1
Surgical Post-operative	170	46	Up to 2500	46	0	0	0
Acute Renal Failure	9	6	Up to 940	6	0	0	0
Self-poisoning	4	3	Up to 960	3	0	0	0

one random sample was taken from each patient, raised total CK activity could have been missed in some patients. Therefore, the frequency of abnormal total CK activity in this mixed surgical group may be an underestimate of the true incidence of this finding.

Class 3: Patients on haemodialysis with acute renal failure.

Two blood samples were taken from each patient. Six of the 9 patients had raised total CK activity, ranging from 220 to 940 IU/l. The MB isoenzyme was not detected in any of these patients.

Class 4: Patients in coma due to barbiturate overdosage.

Three of the 4 patients had abnormal total CK activity (205, 960, 732 IU/l), but no MB isoenzyme activity was detected in any patient.

Class 5: Miscellaneous. There were 2 such patients, one with hypothermia and the other with the "Munchausen Syndrome".

The patient with hypothermia had a very high serum total CK activity of 5100 IU/l but with no MB isoenzyme present.

The patient with the "Munchausen Syndrome" was admitted to the Coronary Care Unit with a severe chest pain, and was found to have a serum total CK activity of 3400 IU/l but with no MB isoenzyme present. Other enzymes (AST and USLD) were normal. His ECG gave no evidence of a recent infarct. The same symptoms had occurred several times in the past. No apparent reason was known for his abnormal CK activity. It is not clear whether the rise in CK activity was related to his long walk in the cold from his previous hospital to the Royal Infirmary, Edinburgh.

Discussion.

The present study shows that the release of the MM isoenzyme of creatine kinase after myocardial infarction differs from the MB isoenzyme in two important aspects, in its pattern of release and in its diagnostic specificity.

The pattern of release of the isoenzymes. The MB isoenzyme had been shown to reach its peak value earlier and to be cleared from blood much faster than the MM isoenzyme. The reasons for the shorter time in reaching peak activity for the MB isoenzyme are not clear, but it might be due to its shorter half-life in blood and its higher myocardium/blood concentration ratio; it cannot be explained in terms of difference of molecular weight or subcellular distribution pattern. The faster clearance rate of the CK isoenzyme might be attributable to the presence of the less stable B subunit in the enzyme molecule. If this were the case, it would be consistent with the fact that the BB isoenzyme is seldom detected in the serum, although the blood-brain barrier could be a contributing factor, the BB isoenzyme being largely present in the brain.

The diagnostic specificity of the isoenzymes. The present study shows that the MB isoenzyme has a better diagnostic specificity for myocardial infarction. In 90 of the 92 patients thought to have had a myocardial infarction, the MM and MB isoenzymes of CK, AST and USLD all showed abnormal results. In the remaining 2 cases, the MB isoenzyme was the only enzyme with raised activity in serum. Moreover, of the 3 cases where the MB isoenzyme was elevated without

myocardial infarction, two were attributable to myocardial damage, whereas there were 6 cases which had raised MM isoenzyme activity with no apparent myocardial involvement (Table 4-7).

The better specificity of the MB isoenzyme is further supported by the study on surgical patients. All the cardiac bypass patients had raised total CK activity in serum, and 12 of them had detectable MB isoenzyme bands. Although the ECG and clinical symptoms of these 12 patients were not conclusive, it was thought likely that the MB isoenzyme came from the myocardium as a result of physical trauma to myocardial cells at the time of the operation. However, it was impossible to be sure that the raised enzyme levels did not reflect enzyme release from areas of ischaemic or infarcted myocardium resulting from thrombi or emboli in coronary vessels. The fact that 46 of the 170 non-cardiac postoperative patients had raised MM isoenzyme activities showed that the use of MM isoenzyme, or total CK, in diagnosing myocardial infarction could be very misleading in patients who had undergone non-cardiac surgery. This in general agrees with the report of Dixon et al., (1973). The MB isoenzyme, absent in all the non-cardiac postoperative patients, is thus the single enzyme activity measurement that is currently available which is capable of suggesting a diagnosis of myocardial infarction in postoperative patients.

The reason for the raised MM isoenzyme activity in serum after surgery is not clear, although it seems possible



that it arises from surgical trauma to muscle at the site of operation. However, no statistically significant relationship has been found between the extent of the postoperative increase and the amount of muscle trauma (Hobson et al., 1972) or the type of operative procedure (Dixon et al., 1971). The nature and duration of anaesthesia prior to operation has, on the other hand, been reported to be closely related to the increase in the serum MM isoenzyme activity (Phornphutkul et al., 1974). Renal dialysis and drug overdosage also cause a rise in serum MM isoenzyme activity, but no rise in MB isoenzyme activity has been detected.

It is worth noting that, although the MB isoenzyme is probably the most specific diagnostic enzyme for myocardial infarction currently available, it is not completely specific for the myocardium. There are a number of non-cardiac situations where the MB isoenzyme is also detected in the serum. For example in muscular dystrophy, the skeletal muscle CK isoenzyme pattern becomes atypical and reverts to a foetal state, with a greater proportion of the MB isoenzyme, and the MB isoenzyme is occasionally observed in the serum of these patients (Goto et al., 1969). Moreover, in the present study, the MB isoenzyme activity was detected in an apparently normal man with a serum CK activity of 400 IU/l.

#### Creatine kinase isoenzymes as indicators of infarct size.

Currently, there is great interest in using a "heart-specific" enzyme to assess the size of a myocardial infarct and hence to predict prognosis and to evaluate therapy.

It has been shown that there is a general parallel between infarct size and the peak values of AST, LD, and total CK (Nydic et al., 1955; Kibe and Nilsson, 1967; Shell et al., 1971). In view of the better heart-specificity of the MB isoenzyme, it seems that the MB isoenzyme might give a better estimate of the infarct size. However, the present findings show that the peak activity of the MB isoenzyme does not correlate satisfactorily with the MM isoenzyme, total CK or total AST, and the MB isoenzyme percentage of the total CK activity was very variable at peak activities of the isoenzyme (Tables 4-3 and 4-4). This suggests that the peak values for serum MB isoenzyme activity might not be a good indicator of infarct size. Table 4-6, on the other hand, shows that the MB isoenzyme could be a better indicator of post-infarct heart failure, which may be regarded as a phenomenon related to infarct size, than the MM isoenzyme. These two apparently contradictory findings could be explained by the possibility that the amount of the MB isoenzyme released per unit time is highly variable. This seems to suggest that peak activity measurements are not a satisfactory method of assessing infarct size as small peak values might come from a big infarct with a slow but continuous release of the enzyme. Therefore, provided low activities of the MB isoenzyme can be measured more sensitively and there is little local intercellular inactivation of the isoenzyme prior to entering the circulation, the total amount of the isoenzyme released into the blood after the infarct may well be a better measurement than total CK activity for assessing infarct size, as suggested by Wagner et al. (1973).

It should be noted that, even if an ideal enzyme completely specific to heart and released only when the cell dies were to be found, assessment of infarct size by means of serum enzyme activity measurements can at best be only semi-quantitative, as the kinetic parameters essential to the calculation are very difficult to obtain accurately. Parameters for the enzyme clearance rate, the extent of inter-cellular inactivation and the enzyme distribution volume could vary widely between individuals as well as with the clinical state. A better estimate of infarct size might, however, be obtained if the enzyme data were to be interpreted in conjunction with changes in haemodynamic cardiac functions (Mathey et al., 1974).

The MB isoenzyme in the diagnosis of myocardial infarction.

Measurement of the activity of the MB isoenzyme can detect myocardial infarction earlier, is more sensitive and more specific for the myocardium, and possibly a more precise indicator of infarct size than measurement of the MM isoenzyme or total CK activity. The facts strongly suggest that the MB isoenzyme is a better diagnostic enzyme for the disease. Since it is present only in very small quantities in normal serum and is usually not detectable by electrophoresis, a qualitative assessment of the isoenzyme is sufficient if a mere confirmation of myocardial infarction is required. However, more valuable clinical information is likely to be obtained if the isoenzyme can be assayed quantitatively. With a sensitive assay technique, a small rise of the MB isoenzyme could indicate a small infarct

which might otherwise escape attention, although proof of this hypothesis is still lacking. Also serial quantitative measurements of the MB isoenzyme activity give a better chance of detecting reinfarction than MM isoenzyme measurement, as the MB isoenzyme has a faster clearance rate. It might also allow a more accurate assessment of infarct size. Hence, it may be important to both short-term and long-term management of the patients.

The major disadvantage of the MB isoenzyme is its short half-life. Patients admitted 36 hours after an infarct may no longer have detectable amounts of the isoenzyme in their serum. The next disadvantage is technical. So far there is still no simple and precise method of measuring this isoenzyme. The present electrophoretic method is far too cumbersome to be adopted routinely. To make full use of the potential clinical value of this isoenzyme, this technical obstacle has to be overcome.

#### Conclusion.

Of all the enzymes studied, cAST, mAST, cMD, mMD, GLDH, USLD, MB isoenzyme of CK, MM isoenzyme of CK and total CK, the MB isoenzyme of CK has been shown to be the most sensitive and specific enzyme test for myocardial infarction provided the blood specimen is taken between 12-30 hours after the onset of symptoms. It is the only enzyme in this study that can reliably help to diagnose myocardial infarction after surgery and trauma.

The quantitation of the MB isoenzyme in serum might enable detection of small infarcts and the occurrence of reinfarction. There are, however, technical problems relating to its accurate assay. To search for a convenient and accurate method of assaying the isoenzyme activity, a better understanding of the biochemical properties of the MM and MB isoenzymes is necessary. This will be the subject of the following sections.

SECTION 5

BIOCHEMICAL STUDIES OF THE MM AND MB  
ISOENZYMES OF CREATINE KINASE

Most of the work on creatine kinase in the past was done with either the MM or the BB isoenzymes, assuming the hybrid form (MB) to have properties intermediate between its parent forms. In most of these investigations the enzymes were extracted from rabbit, chicken or ox tissues. The human isoenzymes have so far been relatively little studied. With the appreciation of the clinical value of the MB isoenzyme in the diagnosis of myocardial infarction (Section 4) it was considered to be of importance to investigate the properties of this isoenzyme and to compare them with those of the other isoenzyme (MM) in the human myocardium. The present section aims to do this.

#### 5-1 Purification of the cytoplasmic isoenzymes from human heart tissue.

Introduction. Creatine kinase was first obtained in crystalline form from rabbit muscle (Kuby et al., 1954). Subsequently, pure preparations of the MM isoenzyme have been obtained from a wide variety of animal tissues - ox, chicken, mouse, human and monkey. Purification of the muscle MM isoenzyme usually presents little problem. It is readily extractable at low ionic strength and may represent as much as 10 - 20% (w/v) of the soluble sarco-plasmic protein of the muscle (Czok and Bucher, 1960; Gosselin-Rey and Gerday, 1970).

The purification of the BB isoenzyme from brain tissue came later than that of the muscle enzyme (Wood, 1963;

Keutel et al., 1968). The method involved is more complex as the isoenzyme usually constitutes less than 1% of the brain cytoplasmic protein (Keutel et al., 1968). Unlike the MM isoenzyme, the BB isoenzyme requires thiol groups to maintain it in a stable state.

Most of the purification procedures in the past took advantage of the unusual stability of both isoenzymes in ethanol. In the presence of the divalent cations  $Mg^{2+}$ ,  $Mn^{2+}$ ,  $Zn^{2+}$ , the enzyme could often be fractionated sharply from other proteins by ethanol. This procedure was then followed by ammonium sulphate precipitation, column fractionation on phosphocellulose and DEAE cellulose, and finally by crystallisation of the enzyme.

In the present purification, the main concern was to separate the two cytoplasmic isoenzymes (MM and MB) from each other; so the following simplified procedure was adopted.

Purification procedure. Unless stated otherwise the whole procedure was carried out at 4°C.

1. Homogenisation. The fat was trimmed from normal human heart tissue obtained at autopsy within 24 hours of death. The tissue (18 g) was cut into small pieces with scissors, suspended in 80 ml 0.25 mol/l sucrose solution at pH 8.0 containing 10 mmol/l KCl, 1 mmol/l  $\beta$ -mercaptoethanol and 1 mmol/l EDTA, and homogenised in a hand-driven glass homogeniser. During homogenisation the pH was maintained at 8.0 with NaOH. The homogenate was filtered through four layers of cheese cloth.



2. Centrifugation. The filtered homogenate was spun in an ultracentrifuge (MSE Superspeed 50) for one hour at 90 000 g to remove the particulate material.
3. Ammonium sulphate precipitation. After centrifugation, solid ammonium sulphate was added to the supernatant to give 40% saturation, the pH being adjusted to 8.0. After spinning, the precipitate was discarded and ammonium sulphate was added to the supernatant to give 75% saturation. The precipitate from this step was redissolved in 25 mmol/l tris/HCl pH 8.0 containing 1 mmol/l EDTA and 1 mmol/l  $\beta$ -mercaptoethanol.
4. Dialysis. The solution was dialysed against the above buffer for 24 hours with two changes of buffer.
5. DEAE cellulose column fractionation. About 120 g DEAE cellulose (Whatman microgranular, preswollen DE 52) was suspended in 100 mmol/l tris/HCl pH 8.0 containing 1 mmol/l  $\beta$ -mercaptoethanol and 1 mmol/l EDTA. Fines were removed, the suspension was degassed and the pH was then adjusted to 8.0. The slurry was packed into a column (14 cm x 5 cm) and then equilibrated with 25 mmol/l tris/HCl pH 8.0 (containing 1 mmol/l  $\beta$ -mercaptoethanol and 1 mmol/l EDTA) by eluting with the buffer until the conductivity and pH of the eluate were the same as the starting buffer.

The dialysed heart extract was then fed into the column by gravity and eluted with the starting buffer. The absorbance of the eluate was monitored at 258 nm using an LKB Uvicord (No. 4701A); fractions were collected on an LKB UltroRac fraction collector (No. 7000). When no more peaks

were observed on the UV-recorder, elution by a linear ionic gradient was started. The gradient was formed by two cylindrical flasks, one containing 500 ml 25 mmol/l tris/HCl pH 8.0 with 1 mmol/l  $\beta$ -mercaptoethanol and 1 mmol/l EDTA; the other contained 500 ml of the buffer with 0.5 mol/l NaCl. Elution was stopped when all the MB isoenzyme was eluted (Fig. 5-1).

The protein concentration of the solution at each purification step was determined by the biuret method (Gornall et al., 1949) with the exception of the column eluates which were measured by absorbance at 280 nm (Warburg and Christian, 1941). Creatine kinase activity was assayed by the method of Rosalki (1967).

Figure 5-2a and b, and Table 5-1 show the results of the purification. The specific activity of the MM isoenzyme showed an overall increase of 94 times with a total activity of 68 000 IU/l; that of the MB isoenzyme was increased 76 times with a total activity of 15 000 IU/l.

Figure 5-1 shows a third peak of about 1.6% of the total creatine kinase activity, occurring mid-way between the MM and MB isoenzyme peaks. Following electrophoresis on a 7% polyacrylamide gel, this enzyme showed the same anodal mobility as the MM isoenzyme peak. It was considered that this small peak of creatine kinase might come either from the small amount of the MM isoenzyme not eluted with the first peak or from cellular particles (probably mitochondria) ruptured during initial homogenisation.

Figure 5-1

DEAE Cellulose Column Fractionation of Dialysed Human Heart Extract

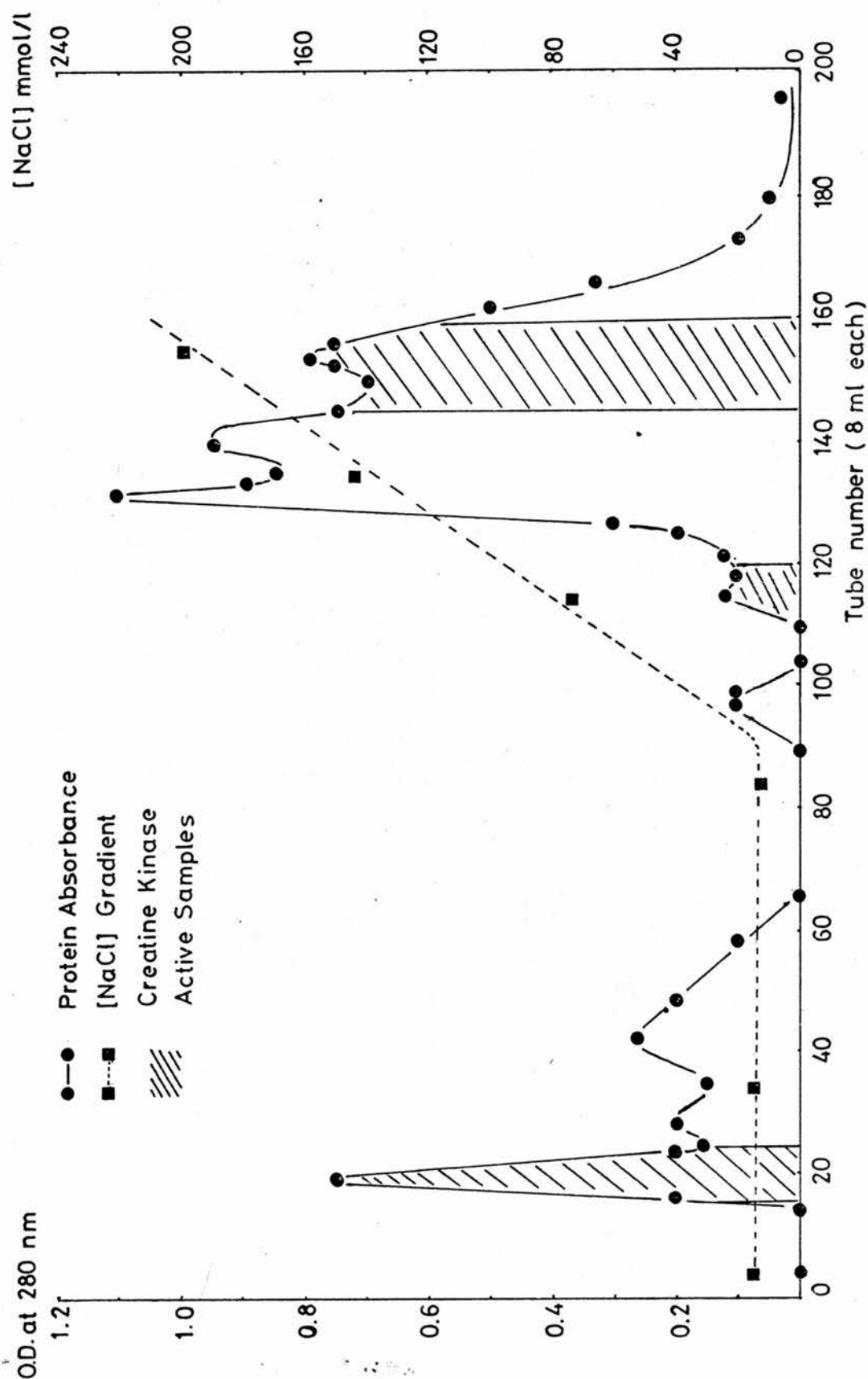
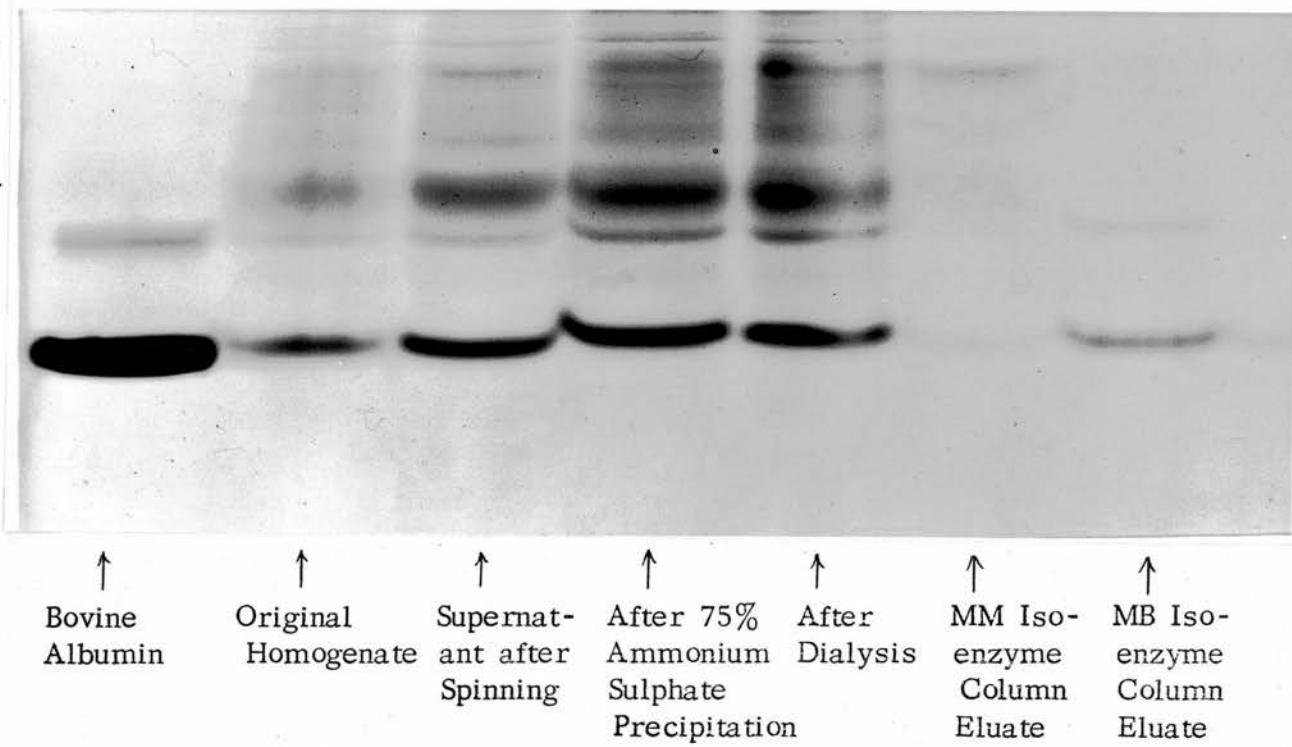


Figure 5-2

Purification of the MM and MB Isoenzymes of Creatine Kinase

a. Electrophoresis of Various Stages of Purification in 7% Polyacrylamide Gel Slab: Stained for Protein with Amido Black



b. Electrophoresis of Various Stages of Purification in 7% Polyacrylamide Gel Slab: Stained for CK Isoenzymes

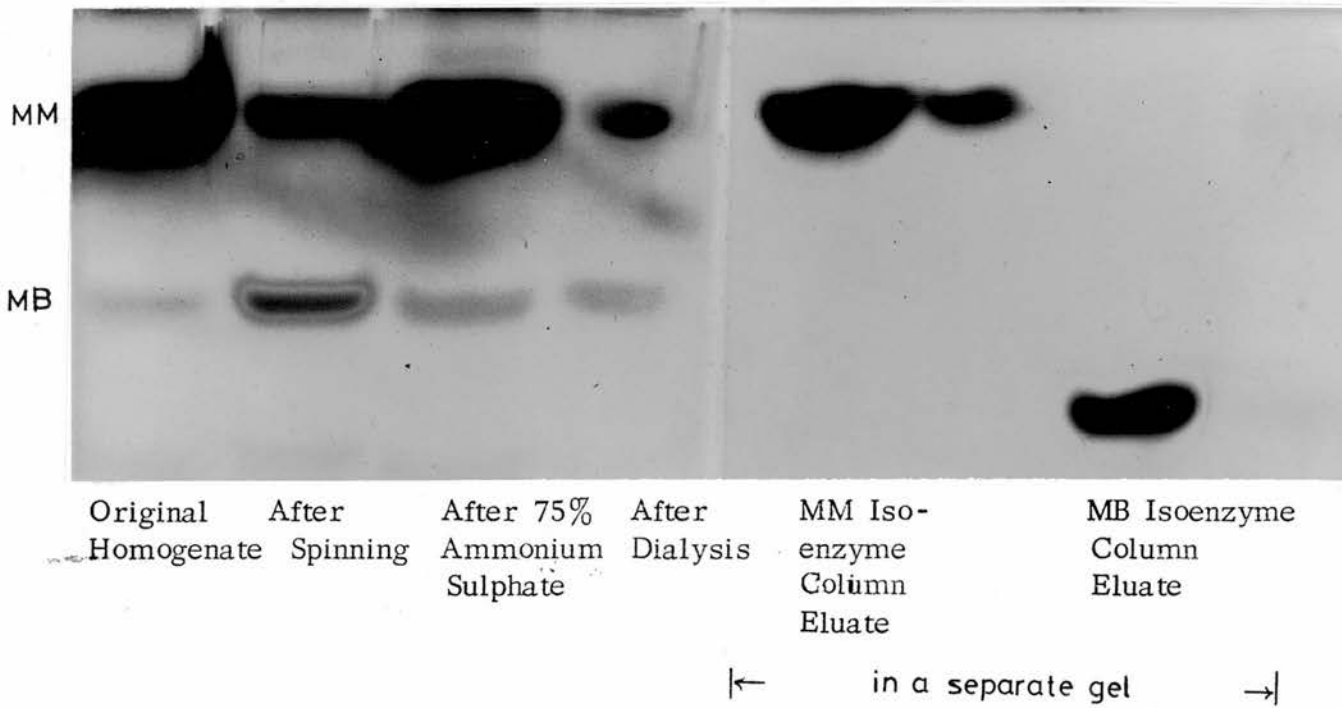


Table 5-1

Purification of Creatine Kinase Isoenzymes

	Total Enzyme Activity		Total Protein (mg)		Specific Activity		Purification		Yield %	
	IU				IU/mg					
	* MM	* MB	MM	MB	MM	MB	MM	MB	MM	MB
Homogenate	14280	6530	7900		1.81	0.83	1.0	1.0	100	100
Supernatant	9510	4350	550		17.30	7.9	9.6	9.6	67	67
75% (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>										
Precipitate	7080	2250	420		16.90	5.4	9.3	6.5	50	34
Dialysis	7600	2410	337		22.60	7.2	12.5	8.6	53	37
DEAE										
Cellulose	5810	1860	34.0	30.3	171	62.7	94.0	76.0	41	29

\* Activity of the individual isoenzymes was obtained from electrophoresis on polyacrylamide gel slab.

All the following studies were done with the iso-enzymes obtained from the first and the last peaks in the column elution, identified electrophoretically as the MM and MB isoenzymes respectively.

Tests for purity. The proportion of MB isoenzyme activity to total activity at various stages of purification was measured by electrophoresis on 7% polyacrylamide gel slabs followed by staining and densitometric scanning (Section 6). Undiluted samples were used for the final column eluates. The percentages of the MB isoenzyme were found to be 30%, 34% and 24% in the original homogenate, centrifuge-super-natant and dialysed extract respectively. For the column eluates containing the partially purified isoenzymes, no activity due to the other isoenzyme was observed in the polyacrylamide gel slab when samples of the MM and MB extracts with activities as high as 6800 and 5500 IU/l respectively were applied. Thus, each of the two partially purified isoenzyme preparations was practically free from contamination by the other. The polyacrylamide gel method was capable of demonstrating creatine kinase activity as low as 5 IU/l (Section 6). This implied that the cross-contamination of each isoenzyme was less than 1 in 1100.

The activities of adenylate kinase and Mg-activated ATPase, which might have interfered with the assay techniques and gel-staining procedure, were measured in the final extracts of both isoenzymes. The adenylate kinase activity was measured at its optimal pH (7.5) with reagents the same as those in the methods of Rosalki (1967), and Hughes (1962),

but omitting creatine phosphate. The ATPase activity was determined by the methods of Tanzer and Gilvarg (1959) and Kuby (Kuby et al., 1954) in the absence of creatine at pH 7.0 and 9.0. In all cases, undiluted enzyme extracts were used. No activity of adenylate kinase or Mg-activated ATPase was detected in either isoenzyme extract. Activities (assayed by methods described in Section 2) of aspartate aminotransferase (5100 IU/l) and malate dehydrogenase (6000 IU/l) were, however, found in the MM isoenzyme preparation.

Stability of the isoenzyme preparations. The MM and MB isoenzyme extracts were stored separately in brown bottles at 4°C. The MM isoenzyme extract was stored in 25 mmol/l tris/HCl at pH 8.0 containing 1 mmol/l  $\beta$ -mercaptoethanol and 1 mmol/l EDTA; the MB extract was stored in an identical solution apart from the presence of NaCl (about 0.2 mmol/l). During the period of study both the activity and the electrophoretic pattern of the extracts were checked about once every four weeks. No significant change in activity was observed and the electrophoretic pattern remained constant throughout the study.

## 5-2 Physical properties of the isoenzymes.

Dawson et al. (1967) compared the molecular weight of the rabbit muscle (MM) and brain (BB) isoenzyme by gel-filtration and sedimentation studies, and showed that both forms had approximately the same molecular weight of around

80 000. Molecular weight determinations of the enzyme subunits indicated that these were half those of the intact molecule (Yue et al., 1968). The molecular weights of purified creatine kinases from various species including humans were also reported to be within the range 78 500 - 85 100 by Watts (1973). Thus, it is fairly certain that the human MM and MB forms have similar molecular weights of about 80 000.

#### 5-2-1      Electrophoretic behaviour.

Since the brain type subunit (B) contains significantly fewer basic amino acids than the muscle type (M) (Eppenberger et al., 1967), the MB isoenzyme has, as expected, a greater anodal electrophoretic mobility than the MM isoenzyme.

The electrophoretic pattern of the MM and MB isoenzymes was studied at pH 8.0 in three different media: a. 7% polyacrylamide gel slab, b. agarose gel and c. cellulose acetate. The enzyme activity was demonstrated by NBT staining in a and c, and NADPH fluorescence in b. (see Section 6 for methods).

On agarose gel and cellulose acetate both the MM and MB isoenzymes migrated as single units, the MM isoenzyme remaining close to the origin and the MB isoenzyme being more anodic (Figs. 5-3 and 5-4b). However, in 7% polyacrylamide gel the MM isoenzyme appeared as three bands near the origin (about the position of  $\gamma$ -globulin) and the MB isoenzyme as two bands midway between the  $\gamma$ -globulin and



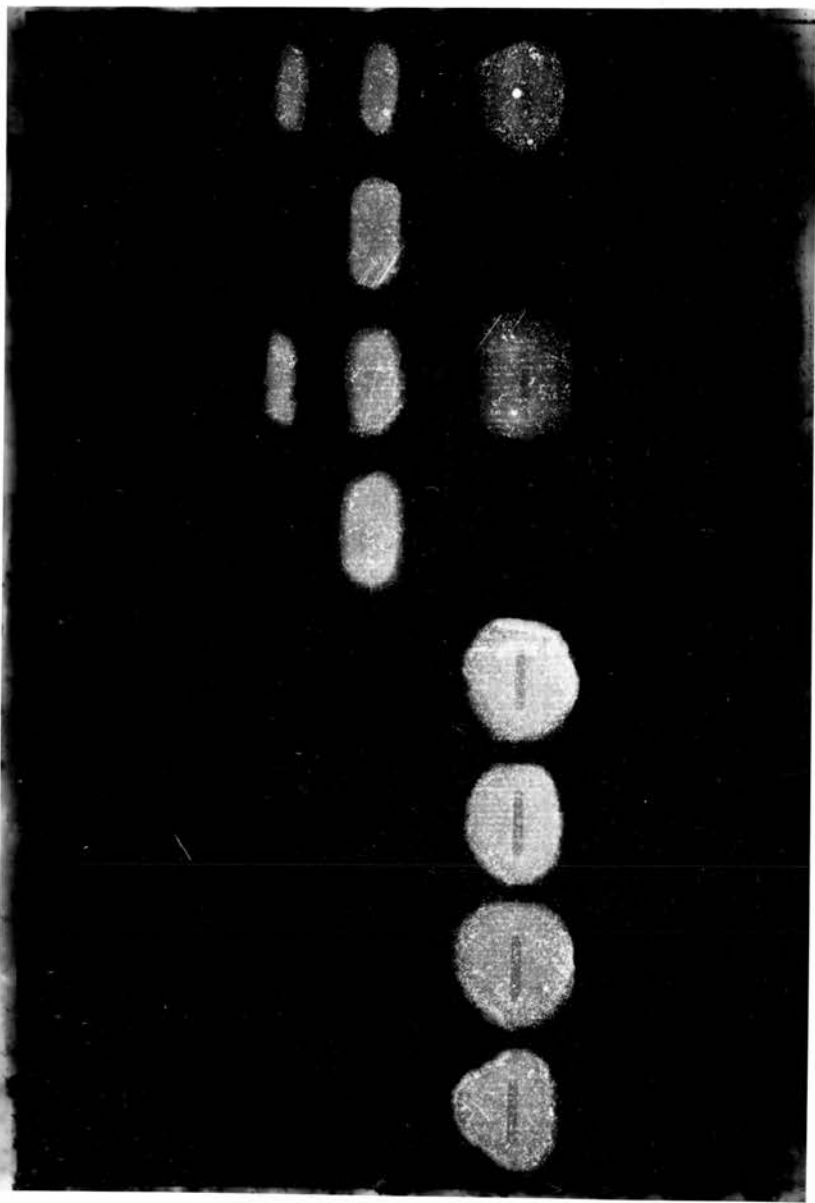
the albumin bands. The same pattern was also seen in sera with elevated creatine kinase activity (Fig. 5-4a).

The explanation for the MM and MB isoenzymes splitting into sub-bands on polyacrylamide gel electrophoresis is not certain. Several explanations are possible:

1. Partial oxidation of the isoenzymes by the ammonium persulphate used to polymerise the gel may have occurred; this is unlikely, however, as the gel has undergone a 45-60 min pre-run during which the small amount of persulphate in the gel would probably be removed.
2. The isoenzyme molecules may have been degraded into different forms during purification. This is also unlikely since the same sub-band pattern was found in serum creatine kinase.
3. The sub-bands might represent different conformational forms of the isoenzymes. If so, however, the MB isoenzyme might have been expected to show more sub-forms than the MM as the B subunit is less compact and hence more likely to assume different conformations.
4. The sub-bands might represent genuine isoenzyme heterogeneity. The fact that this was observed only in polyacrylamide gel could be ascribed to the better resolving power of this medium than either agarose or cellulose acetate. The observed patterns of sub-bands (3 for the MM and 2 for the MB isoenzyme) is compatible with the presence of two "M" type subunits, M and M', giving rise to MM, MM', M'M', MB and M'B.

Figure 5-3

Dissociation of the Sub-units of MM and MB Isoenzymes  
(Shown on Agarose Film after Electrophoresis)



BB Isoenzyme →

MB Isoenzyme →

MM Isoenzyme →

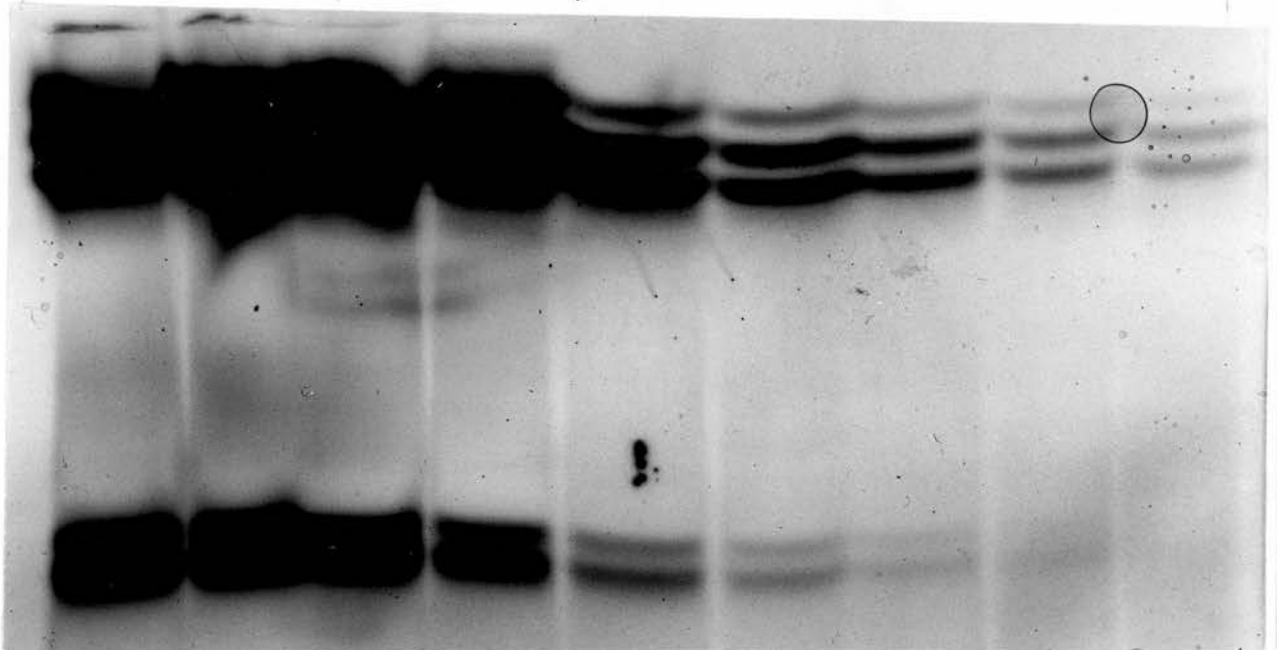
Weak Cationic →  
Band (Not  
Apparant Here  
due to Fading)

MM Control   MM Urea   MM Control   MM Guanidine   MB Control   MB Urea   MB Control   MB Guanidine

Figure 5-4

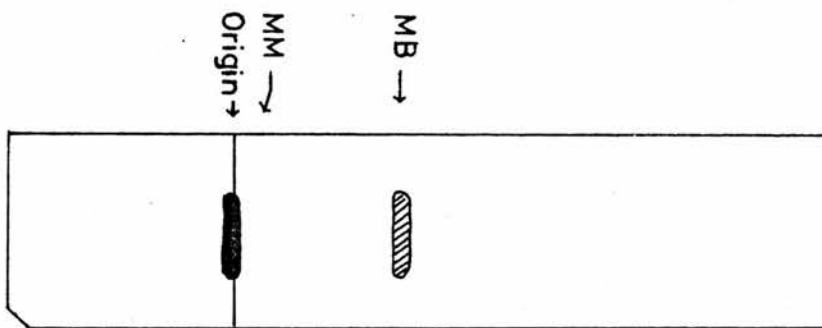
Electrophoresis of The MM and MB Isoenzymes of  
Creatine Kinase

a. On 7% Polyacrylamide Gel Slab (Using Serum Samples)



Sample 1 2 3 4 1' 2' 3' 4' 1''  
' , '' = 2 & 4 fold dilution respectively

b. On Cellulose Acetate (Using Purified Enzyme Extracts)



2  $\mu$ l of the mixture solution of purified MM and MB isoenzymes was applied onto the cellulose acetate strip. Electrophoresis was performed for 45 min in 50 mmol/l veronal buffer pH 8.5. The isoenzyme activities were demonstrated by incubating the strip with a layer of substrate solution containing the necessary coupling enzymes in a manner similar to that in the polyacrylamide gel method (see text).

5-2-2

Dissociation of subunits.

If the MB isoenzyme subunits are separated and allowed to recombine, three forms of isoenzyme - MM, MB and BB, should result, their relative proportions being dependent on the thermodynamic stability of the different subunit associations. If the MM isoenzyme is subjected to the same treatment only one form of isoenzyme (MM) is to be expected. The usual means of subunit dissociation are by treatment with urea, guanidine hydrochloride, low pH or repeated freezing and thawing under appropriate conditions.

The following procedure was carried out at 4°C. 60 IU of MM isoenzyme and 12 IU MB isoenzyme were separately treated with i) 8 mol/l urea and ii) 6.5 mol/l guanidine hydrochloride solutions containing 25 mmol/l tris/HCl pH 7.7 and 0.1 mol/l  $\beta$ -mercaptoethanol. After mixing, the solutions were immediately transferred to dialysis tubes (Visking 8/32) and dialysed against 25 mmol/l tris/HCl containing 0.1 mol/l  $\beta$ -mercaptoethanol for 48 hours. Control experiments, in which the urea and guanidine hydrochloride were omitted, were also performed. After dialysis, agarose gel electrophoresis of the dialysed solutions was followed by demonstration of enzyme activity by NADPH fluorescence (Section 6).

The results for the guanidine hydrochloride and urea treated enzyme preparations were the same. Four bands were observed in the treated MB isoenzyme solutions, corresponding to the MM, MB, BB isoenzymes and a weak cationic band. This may be taken as confirmation that the isoenzyme

isolated in this preparation is the hybrid MB type. In the treated MM isoenzyme solutions, one strong MM and one weak cationic band were detected. The weak cationic band was absent in both MM and MB controls (Fig. 5-3).

The reactivation of the isoenzymes on dialysis after urea or guanidine hydrochloride treatment was very dependent on experimental conditions. Initial experiments showed that reactivation did not occur if i) 0.1 mol/l  $\beta$ -mercapto-ethanol was absent from the dialysing buffer, ii) the incubation mixture was left standing for 30 min at 4°C before dialysis, iii) the isoenzyme preparations were of low activity (about 3 IU) or iv) the procedure was carried out at room temperature (about 22°C).

The nature of the extra cationic band obtained after treatment is uncertain. It may be a result of partial degradation of the M subunit by the dissociating reagents or it may be a reflection of the heterogeneity of the M subunit.

### 5-2-3

#### Inactivation by heat.

The effect of heat on enzyme activity is two-fold. It affects i) the structure of the enzyme molecule, and ii) the kinetics of the enzyme reaction. The present subsection deals with the first effect.

The inactivation of enzymes by heat is nearly always due to the denaturation of the enzyme protein. The rate of the inactivation is dependent on a variety of factors

including the temperature, pH, ionic strength and protein concentration of the solution. It may also be affected by the presence of substrates, inhibitors or other substances which may stabilise the enzyme molecule. In some cases the denaturation of enzyme by heat may be partially reversible on cooling, there being some recovery of enzyme activity.

The stability of creatine kinase isoenzymes towards temperature was studied by i) comparing the inactivation of the isoenzymes at different temperatures, ii) measuring the rate of inactivation at a fixed temperature, and iii) studying the effects of substrates and other substances on the stability of the two isoenzymes.

#### Procedure and Results.

Creatine kinase activity was measured by the Rosalki (1967) method. In all cases the total volume of the enzyme solution for heat treatment was 1.0 ml contained in plastic cuvettes (1 cm internal diameter; 1 mm wall thickness). The enzyme solution contained 2 g protein/ l (adjusted with pure bovine albumin) in 100 mmol/l tris/HCl pH 7.9 (50°C). The precision of the temperature control in the water bath used in these experiments was  $\pm 0.1^{\circ}\text{C}$ .

##### 1. Heat inactivation at different temperatures.

The isoenzyme solutions were incubated between 40°C and 70°C for 15 min. At the end of the incubation 25  $\mu\text{l}$  aliquots were pipetted from the incubating tubes into the assay solutions. Their activities were measured within 30 min after the incubation.

Inactivation became detectable above 38°C for the MB isoenzyme and above 43°C for the MM isoenzyme. Above 60°C there was complete inactivation of both isoenzymes (Fig. 5-5).

The pH of the incubation solutions was found to vary from pH 7.7 at 60°C to pH 8.3 at 40°C. Although both isoenzymes were stable within this pH range at 37°C, the observed differences in heat inactivation might be partly due to this indirect effect of variation in pH.

## 2. Rate of inactivation at 50°C.

The rates of inactivation of four isoenzyme preparations were studied:- i) 100% MM, ii) 50% MM + 50% MB, iii) 25% MM + 75% MB and iv) 100% MB. The logarithm of the percentage of residual activity was plotted against the incubation time and half-lives ( $T_{\frac{1}{2}}$ ) for the isoenzymes were calculated.

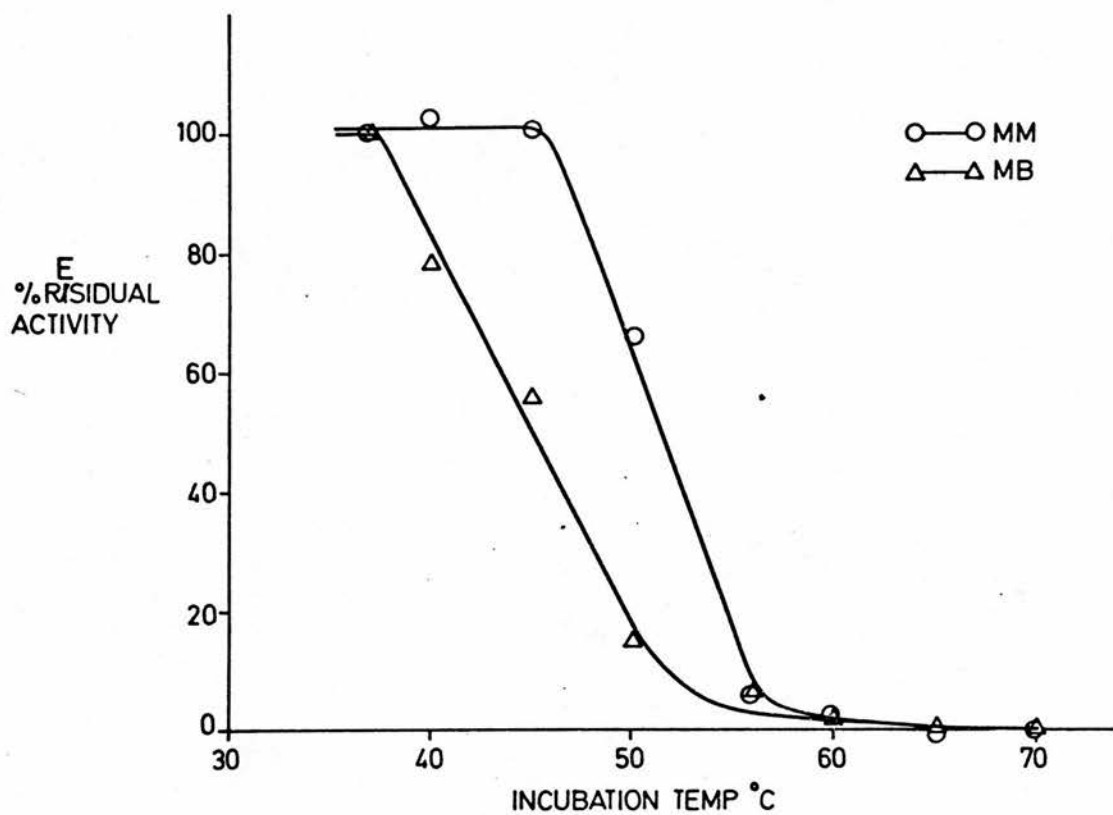
The purified isoenzymes showed a single component logarithmic inactivation rate, whereas the isoenzyme mixtures showed two component inactivation curves, one component corresponding to each of the isoenzymes (Fig. 5-6). The  $T_{\frac{1}{2}}$  life was 27 min for the MM and 5 min for the MB isoenzymes.

## 3. Effects of substrates and other substances at 50°C.

The substrates in concentrations equal to their  $K_m$  values (Section 5-3-2) were incubated with the isoenzyme solutions in 100 mmol/l tris/HCl pH 7.9 at 50°C. Samples were removed at various intervals and analysed for activity. The  $T_{\frac{1}{2}}$  values were calculated as in 2. In addition to the enzyme substrates, the effects of  $\beta$ -mercaptoethanol (5 mmol/l),

Figure 5-5

Heat Inactivation of Creatine Kinase Isoenzymes

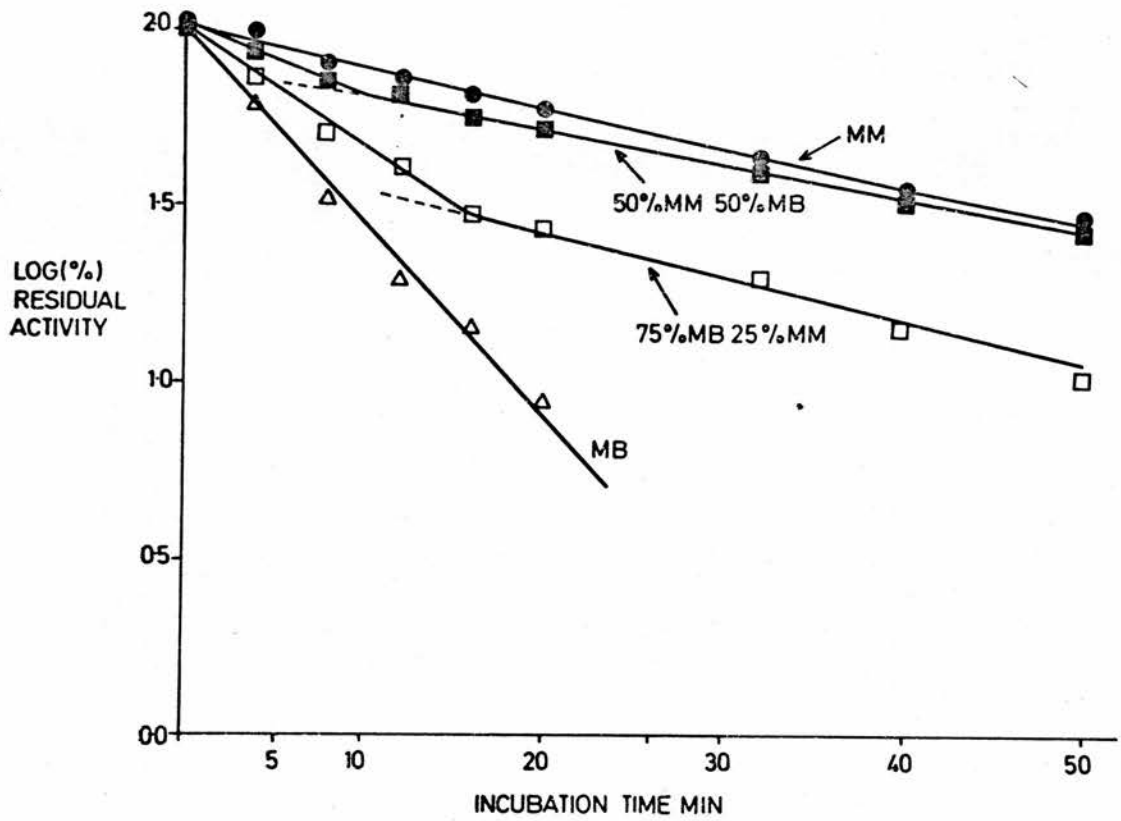


Each isoenzyme was incubated at the given temperature for 15 min.



Figure 5-6

Heat Inactivation of Creatine Kinase Isoenzyme Mixtures  
at 50°C



bovine albumin (2 g/1), magnesium ions (2 mmol/1), ADP (0.25 mmol/1 for MB; 0.5 mmol/1 for MM isoenzyme) and ATP (1.0 mmol/1) were studied.

$\beta$ -mercaptoethanol, albumin,  $\text{MgADP}^-$ ,  $\text{MgATP}^{2-}$  and  $\text{ATP}^{4-}$  protected the MM isoenzyme from heat denaturation, but they were without effect on the MB isoenzyme.  $\text{ADP}^{3-}$ , creatine and creatine phosphate did not protect either isoenzyme. Free  $\text{Mg}^{2+}$  ions caused both isoenzymes to be inactivated more rapidly (Table 5-2).

Summary. In no case was reactivation of the isoenzyme on cooling observed, at least in the first hour after heat treatment. All the results in 1, 2 and 3 show that the MB isoenzyme is more heat labile than the MM isoenzyme. It has a shorter half-life at  $50^\circ\text{C}$ , is inactivated at a lower temperature and is less protected by substrates.

#### 5-2-4

#### Effect of urea.

The inactivation of enzymes by urea is probably the result of protein denaturation caused by breakage of hydrogen bonds, although the exact mechanism is complex. It is usually impossible to define the initial and final states of the denaturation and studies on enzyme inactivation by urea tend to be empirical, subject to the effects of numerous factors.

Procedure. The enzyme activity was measured by the Rosalki (1967) method using a commercial reagent kit obtained from Boehringer Corporation, England.

Table 5-2

Effects of Various Substances on the Heat Stability of Creatine  
Kinase Isoenzymes at 50°C

Reagents	T <sub>1/2</sub> (min)	
	MB Isoenzyme	MM Isoenzyme
Water	5.4	26
β-mercaptoethanol (5 mmol/l)	4.6	60
Bovine Albumin (2 g/l)	4.6	39
Creatine	4.5	21
▲ Creatine phosphate	4.8	21
Magnesium Acetate (2 mmol/l)	3.0	10
*ADP (0.25 mmol/l for MB; 0.5 mmol/l MM)	4.5	26
*ATP (1.0 mmol/l)	4.5	34
▲ <sup>+</sup> ADP + Mg ([Mg <sup>2+</sup> ]/ [ADP] = 5)	5.0	35
▲ <sup>+</sup> ATP + Mg([Mg <sup>2+</sup> ]/[ATP] = 2)	4.8	45

\* At pH 7.9 ADP and ATP existed mainly as ADP<sup>3-</sup> and ATP<sup>4-</sup> respectively.

+ At pH 7.9 the main ionic species for ADP and ATP were MgADP<sup>-</sup> and MgATP<sup>2-</sup> respectively.

▲ The concentrations of all the enzyme substrates were equal to their respective K<sub>m</sub> values.

The incubation mixture contained 20 mmol/l tris/HCl pH 7.5 100 mmol/l  $\beta$ -mercaptoethanol, 0.8 g protein/ l (adjusted with bovine albumin) and urea at various concentrations (2 mol/l, 5 mol/l and 8 mol/l). Suitable amounts of purified extracts of the MM and MB isoenzymes were mixed with the incubation solution to a final activity in the absence of inhibitor of about 700 IU/l. The incubations were at 4°C, incubation in 8 mol/l urea was also at 25°C. 25  $\mu$ l samples were removed at timed intervals and creatine kinase activity measured immediately.

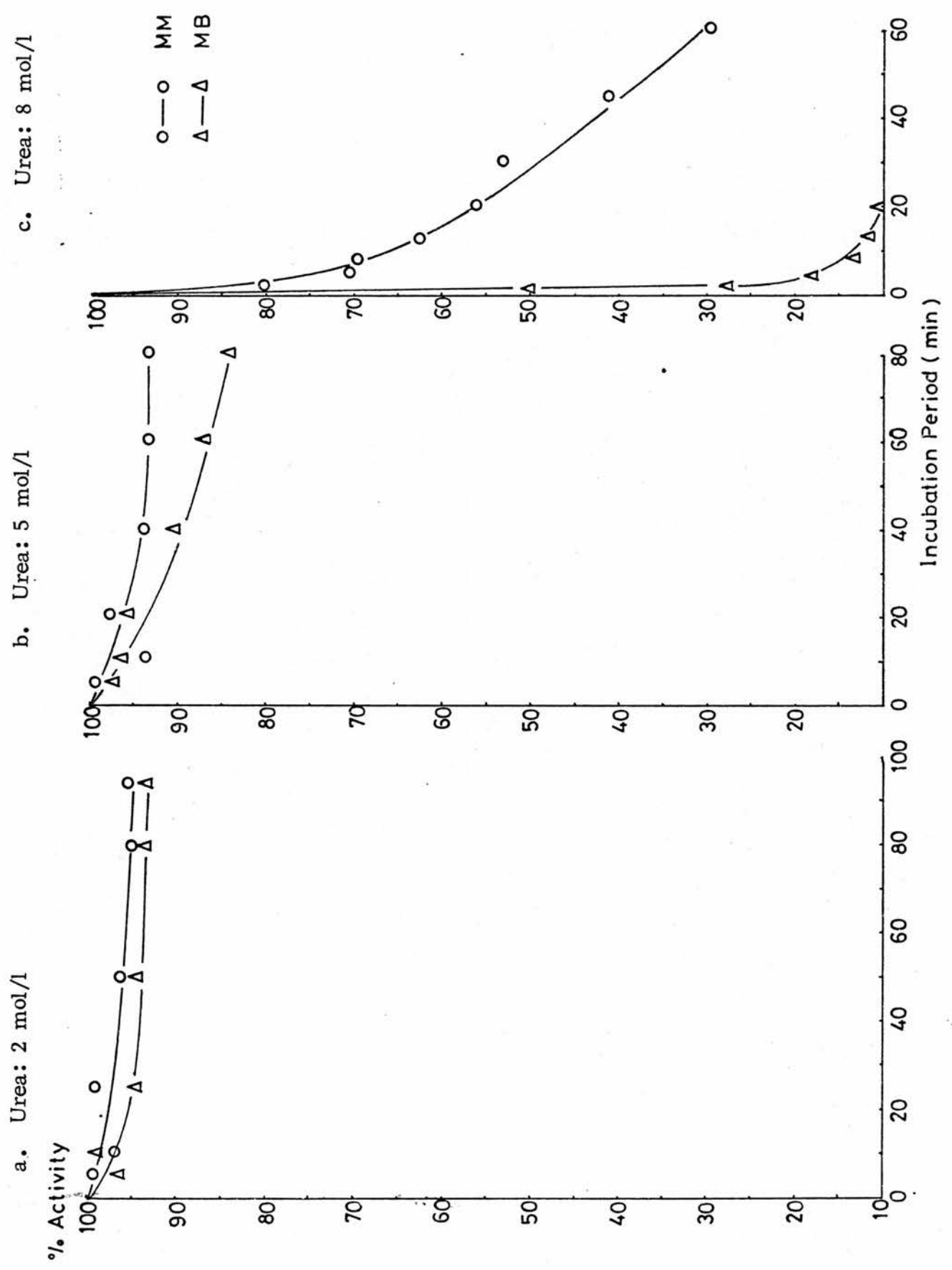
The possible effect of reactivation due to dilution in the assay medium was also investigated. After the enzyme had been incubated with urea for 90 min, 25  $\mu$ l samples of the mixture were delivered into the assay solutions and left standing at 37°C for various intervals. At the end of each interval, substrate was added and the enzyme activity measured.

The effect of urea in the assay solution on the isoenzyme activities was also studied between 0-1.6 mol/l urea. The assay solution was pre-incubated for 2 hours at 25°C and 15 min at 37°C before the activity was measured.

Results. The results are shown in Fig. 5-7. There was little inactivation in 2 mol/l and 5 mol/l urea although the MB seemed to be more affected than the MM isoenzyme in both cases. In 8 mol/l urea both isoenzymes were inactivated, the MB isoenzyme ( $T_{\frac{1}{2}} = 2$  min) much more rapidly than the MM ( $T_{\frac{1}{2}} = 30$  min).

Figure 5-7

Inactivation of Creatine Kinase Isoenzymes by Urea  
at 4°C



The rate of inactivation was temperature dependent (Fig. 5-8). The  $T_{\frac{1}{2}}$  life values of both isoenzymes at 25°C were too short to be measured accurately. The logarithmic plot (Fig. 5-8) shows that the decay curves of both isoenzymes are non-linear suggesting a complex non-first-order inactivation.

For all three concentrations of urea, no reactivation of either isoenzyme was found within 2 hours after urea incubation. This suggests that urea in the stated experimental conditions not only dissociates the subunits of the isoenzyme (which might be expected to re-associate when the urea is greatly diluted - Section 5-2-2), but also affects the tertiary (and possibly secondary) structures of the protein subunits.

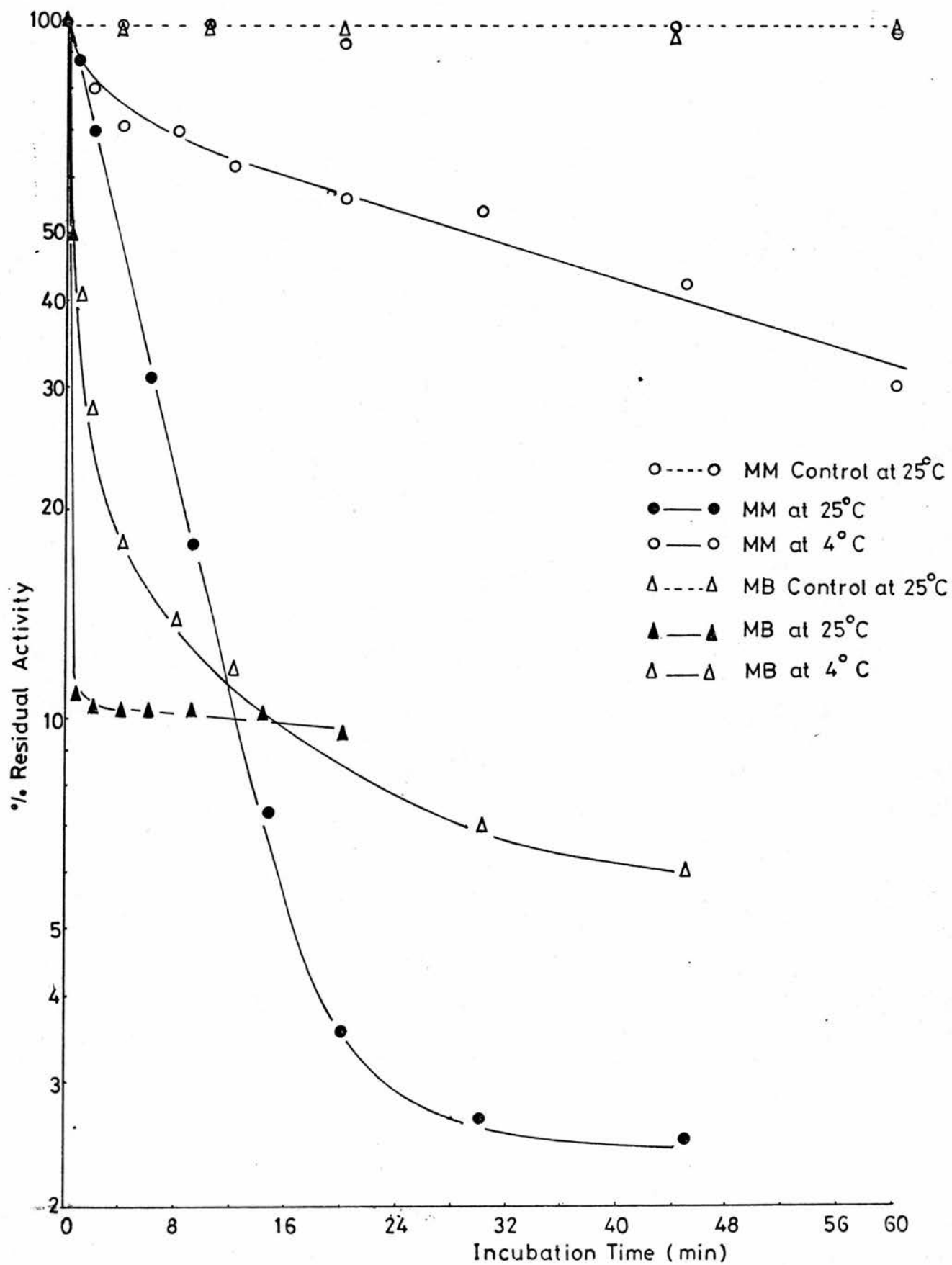
Urea in the assay solution inhibited both isoenzymes to a similar extent; half-activity concentrations were 0.63 mol/l for the MM and 0.69 mol/l for the MB isoenzymes.

#### 5-2-5 Inactivation by thiol-reacting reagents.

Earlier inhibition studies showed that creatine kinase contains thiol groups (Bailey and Perry, 1947). By studying the reaction with iodoacetate and 2-4-dinitro-1-fluorobenzene Mahowald et al. (1962) identified two active cysteine thiol groups in the molecule. Of these two thiol groups, one was found to be present in each of the catalytic sites on the enzyme (Watts et al., 1962). The amino acid sequence associated with these "essential" thiol groups was

Figure 5-8

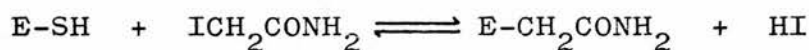
Urea Inactivation of the Isoenzymes of Creatine Kinase at  
Different Temperatures



found to be very similar in both the muscle type and the brain type subunits.

# 1. Iodoacetamide.

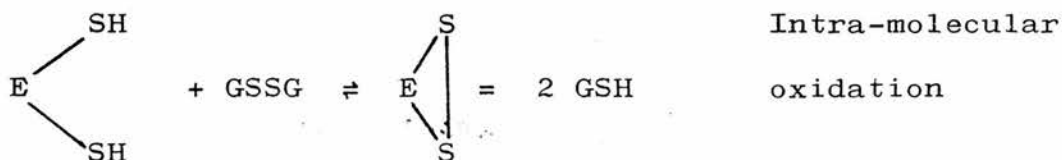
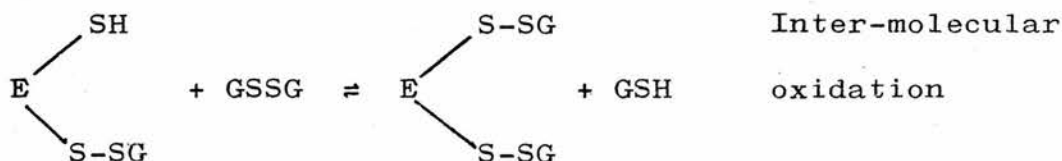
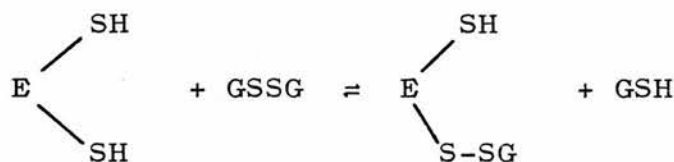
Iodoacetamide is thought to inactivate thiol-containing enzymes by alkylating the thiol groups. However, the inactivation is often complicated by side reactions and secondary effects of alkylation.



In studies on the alkylation of creatine kinase, iodoacetamide is usually preferred to iodoacetate because, while inactivation by both reagents is unaffected by pH variations from 6-9 (Rabin and Watts, 1960), inactivation by iodoacetamide (but not iodoacetate) is unaffected by changes in the ionic strength of the medium (Watts and Rabin, 1962).

# 2. Oxidised glutathione (GSSG).

This disulphide may serve as an oxidant of the enzyme's thiol groups. The extent of the reaction depends on the redox potential difference between glutathione ( $E'_0$  at pH 7.0 = -0.23 volts) and the enzyme's thiol group involved.



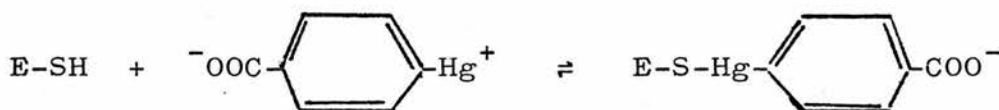


For enzymes with more than one reactive thiol group, both inter- and intra-molecular oxidations are theoretically possible. In practice, however, intra-molecular oxidation is less common, as its occurrence depends on the mutual accessibility of the two thiol groups and their steric orientation.

Disulphide oxidation usually occurs slowly but the rate may be increased by raising the temperature and concentration of GSSG (Hopkins et al. 1938). Increase in pH to about 9.0 favours the oxidation of the enzyme's thiol groups, presumably the  $-S^-$  form reacts more readily.

### 3. p-Hydroxymercuribenzoate (pHMB).

This is a very reactive thiol reagent. The pHMB  $^-OOC-C_6H_4-Hg^+$  ion reacts with thiol groups forming mercaptides:-



#### Procedure and Results.

1. In all experiments creatine kinase activity was measured by the Rosalki method (1967). A sample of the isoenzyme preparation, equivalent to 700 IU/l in the incubation mixture, was incubated with the respective thiol-reacting reagent solution which contained a total protein concentration of 0.8 g/l obtained by adding bovine albumin. The activity of this incubation mixture was assayed at timed intervals by removing 25  $\mu$ l portions. Controls were performed omitting the thiol-reacting reagents.

The incubation mixture in each case contained i) 1 mmol/l

iodoacetamide in 2.0 mmol/l tris/HCl buffer pH 7.0 at 0 - 1°C, ii) 10 mmol/l oxidised glutathione in 2.0 mmol/l tris/HCl pH 9.0 at 20°C, and iii) 14 mmol/l pHMB in 30 mmol/l tris/HCl pH 8.0 at 2 - 3°C.

The logarithm of the percentage residual activity was plotted against the incubation time. The MM and MB isoenzymes were each affected by iodoacetamide to a similar extent (Fig. 5-9a). Oxidised glutathione did not cause significant inactivation of either isoenzyme (Fig. 5-9b) over a period of 100 min. Both the MM and MB isoenzymes were inactivated by pHMB, the MM ( $T_{1/2}$  8 min) more rapidly than the MB isoenzyme ( $T_{1/2}$  60 min) (Fig. 5-9c). The half-lives of the two isoenzymes in the presence of these thiol reagents are listed in Table 5-3.

1. The inhibitory effects of the thiol reagents in the reaction mixture were also studied. The concentrations used were 0-400 mmol/l iodoacetamide, 0-8.0 mmol/l GSSG and 0-6.0 mmol/l pHMB. They were separately pre-incubated in the reaction mixture for 2 hours at 25°C and 15 min at 37°C before activity was measured using the method of Tanzer and Gilvarg (1959).

Iodoacetamide, GSSG and pHMB were all found to be inhibitory (Table 5-4). There were no significant differences between the isoenzymes.

3. Since the greatest difference between the two isoenzymes was found in the inactivation by pHMB, the action of pHMB was chosen for study in more detail. The Rosalki method (1967) was used for all activity measurements.

Figure 5-9

Inactivation of Creatine Kinase Isoenzymes by Thiol-reacting  
Reagents

a. Iodoacetamide ( 1 mmol/l)

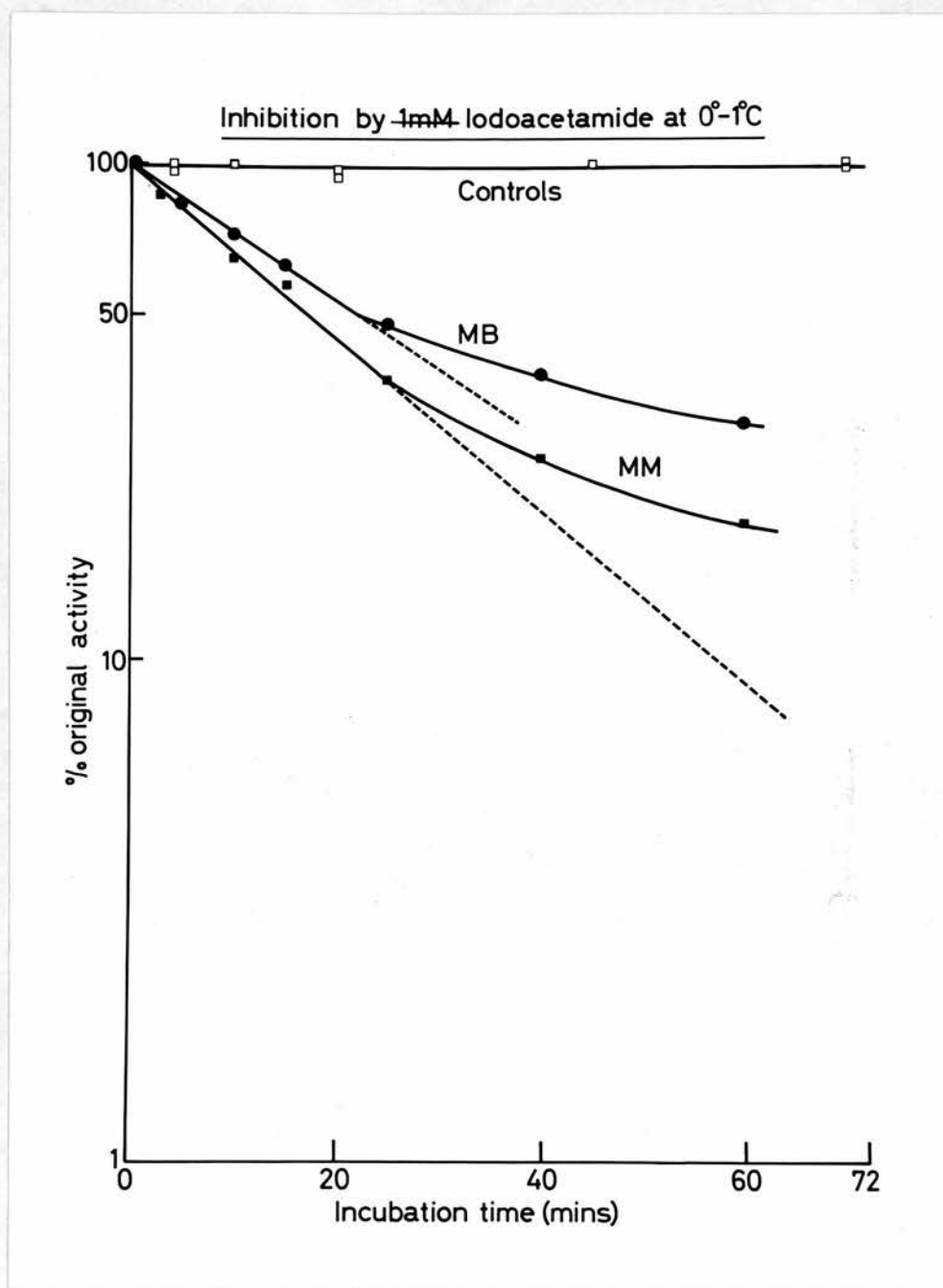


Figure 5-9

Inactivation of Creatine Kinase Isoenzymes by Thiol-reacting  
Reagents

b. Oxidised Glutathione ( 10 mmol/l)

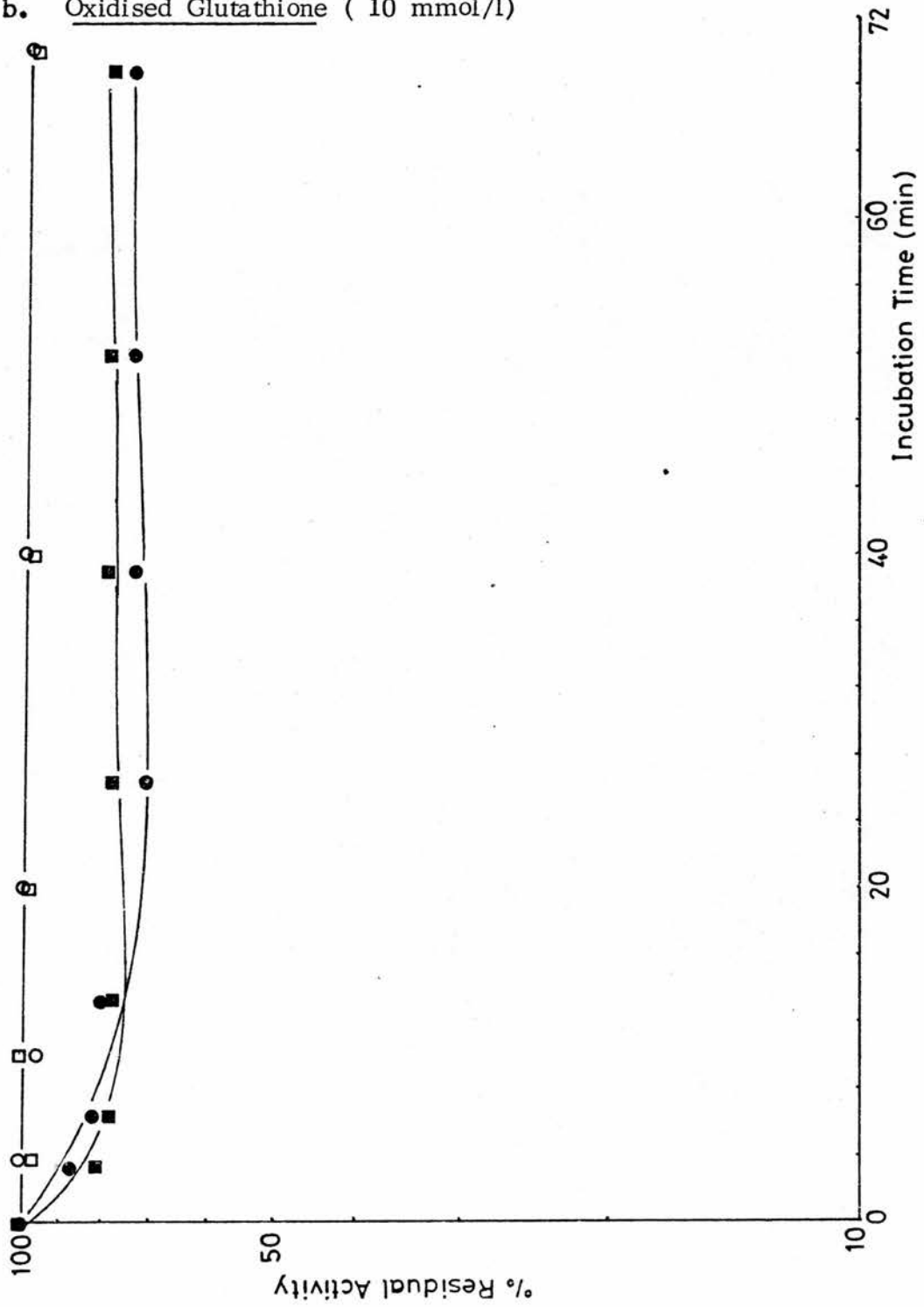


Figure 5-9

Inactivation of Creatine Kinase Isoenzymes by Thiol-reacting  
Reagents

c. p-hydroxymercuribenzoate (pHMB) ( 14 mmol/l)

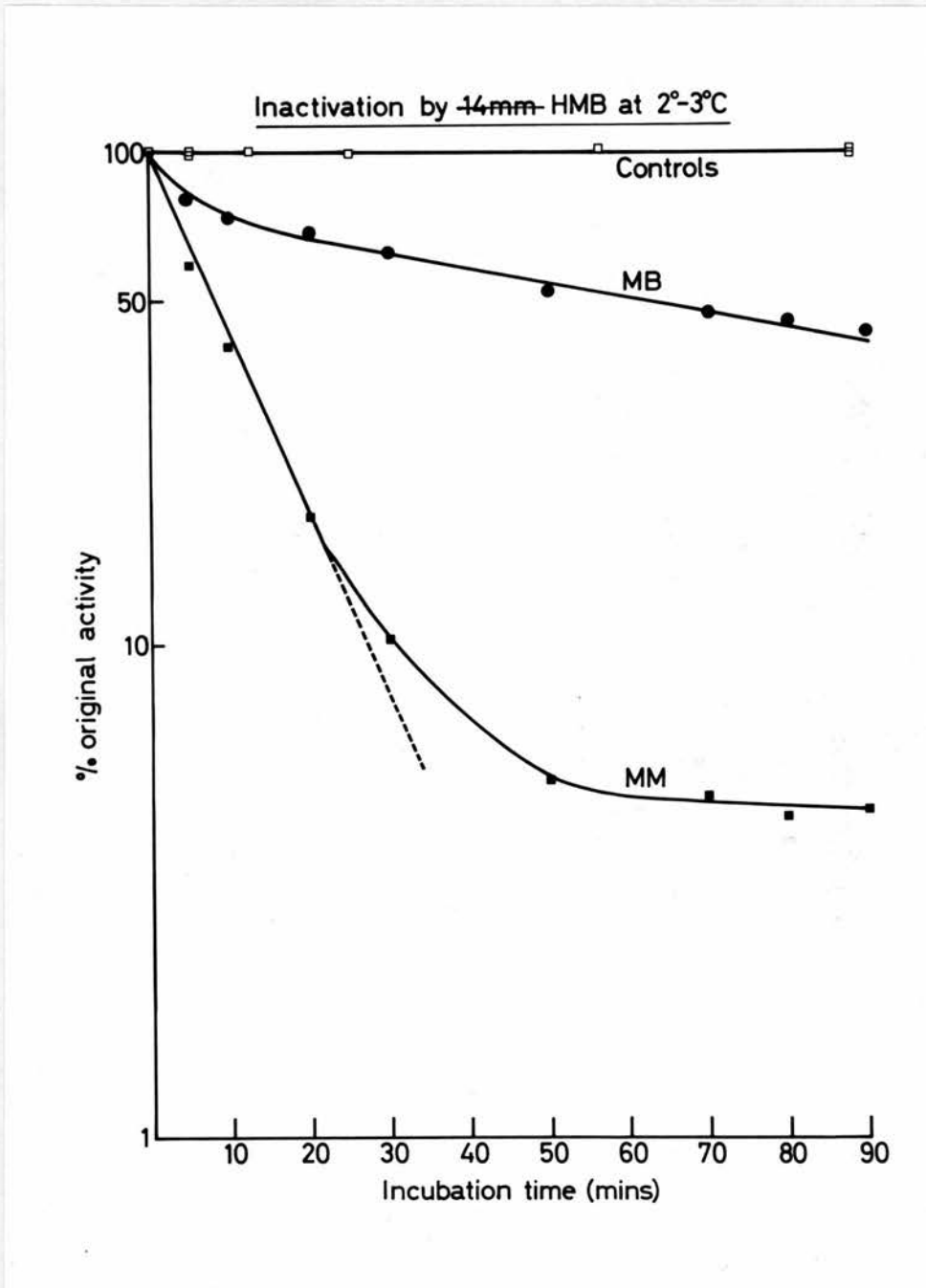


Table 5-3

Inactivation of the MM and MB Isoenzymes by Thiol-reacting Reagents

Reagents	pH	Temperature °C	T <sub>1/2</sub> (min)	
			MB	MM
Iodoacetamide (1 mmol/l)	7.0	0° - 1°	22	17
Oxidised Glutathione (10 mmol/l)	9.0	25°	Very Slow Inactivation	
p-hydroxy- mercuri- benzoate (14mmol/l)	8.0	2° - 3°	60	8

The enzyme activity was measured in the backward reaction by the Rosalki method (1967).

Table 5-4

Inhibition of Activity of Creatine Kinase Isoenzymes

Inhibitors	Concentration for Half-activity Inhibition	
	MM Isoenzyme	MB Isoenzyme
Oxidised Glutathione	3.25 mmol/l	4.85 mmol/l
Iodoacetamide	148 µmol/l	133 µmol/l
p-hydroxy- mercuri- benzoate	2.4 mmol/l	2.6 mmol/l

The enzyme activity was measured in the forward reaction by the method of Tanzer and Gilvarg (1959) with inhibitors in the assay solution containing 5 mmol/l β-mercaptoethanol.

i) Effect of pHMB concentration. The inactivation was carried out over a range of pHMB concentrations (0-20 mmol/l) under the same conditions as in 1. Activity was determined after 90 min incubation.

The MM isoenzyme was more readily inactivated by pHMB than was the MB isoenzyme. The maximal difference occurred in 14-15 mmol/l pHMB (Fig. 5-10a).

ii) Effect of pH. The isoenzymes were incubated in the range pH 7.4 to pH 9.0 with 14 mmol/l pHMB for 90 min, all other experimental conditions being the same as above.

Variation of pH did not affect the pattern of inactivation of the MM isoenzyme; in contrast the MB isoenzyme showed least inactivation at pH 8.0-8.5 (Fig. 5-10b).

iii) Effect of tris/HCl concentration. Keeping the pH at 8.0, pHMB at 14 mmol/l and the incubation period at 90 min, the concentration of tris (added as tris/HCl buffer) was varied from 20 mmol/l to 100 mmol/l. A control experiment was performed with a similar increase of chloride concentration, added as sodium chloride.

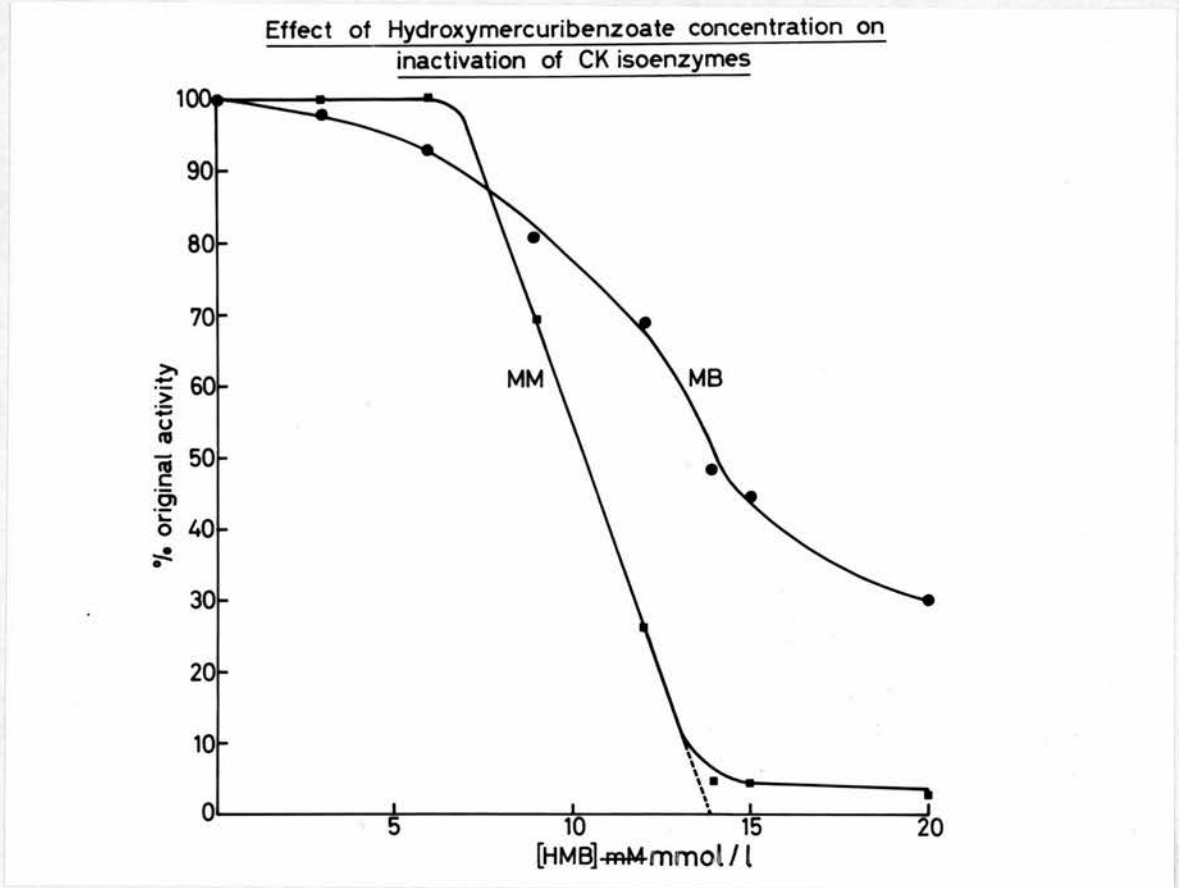
The difference between the rates of inactivation of the two isoenzymes was critically dependent on the concentration of tris/HCl (Fig. 5-10c). The control experiment with chloride ion showed that the effect observed was due to variation in tris concentration.

iv) Buffer types. The isoenzymes were incubated for 90 min with three other types of buffer, 30 mmol/l potassium phosphate, 30 mmol/l triethanolamine and 30 mmol/l veronal,

Figure 5-10

Inactivation of the MM and MB Isoenzymes of Creatine Kinase  
by p-hydroxymercuribenzoate

a.



b.

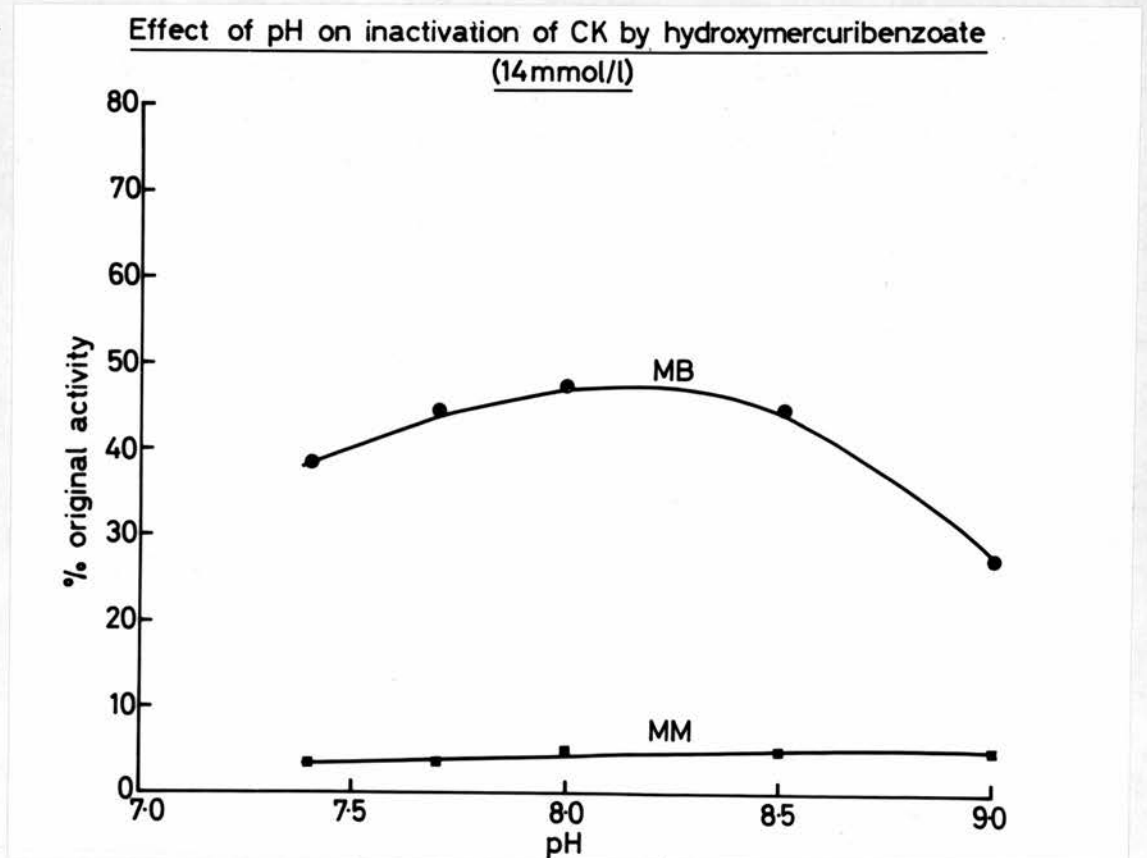
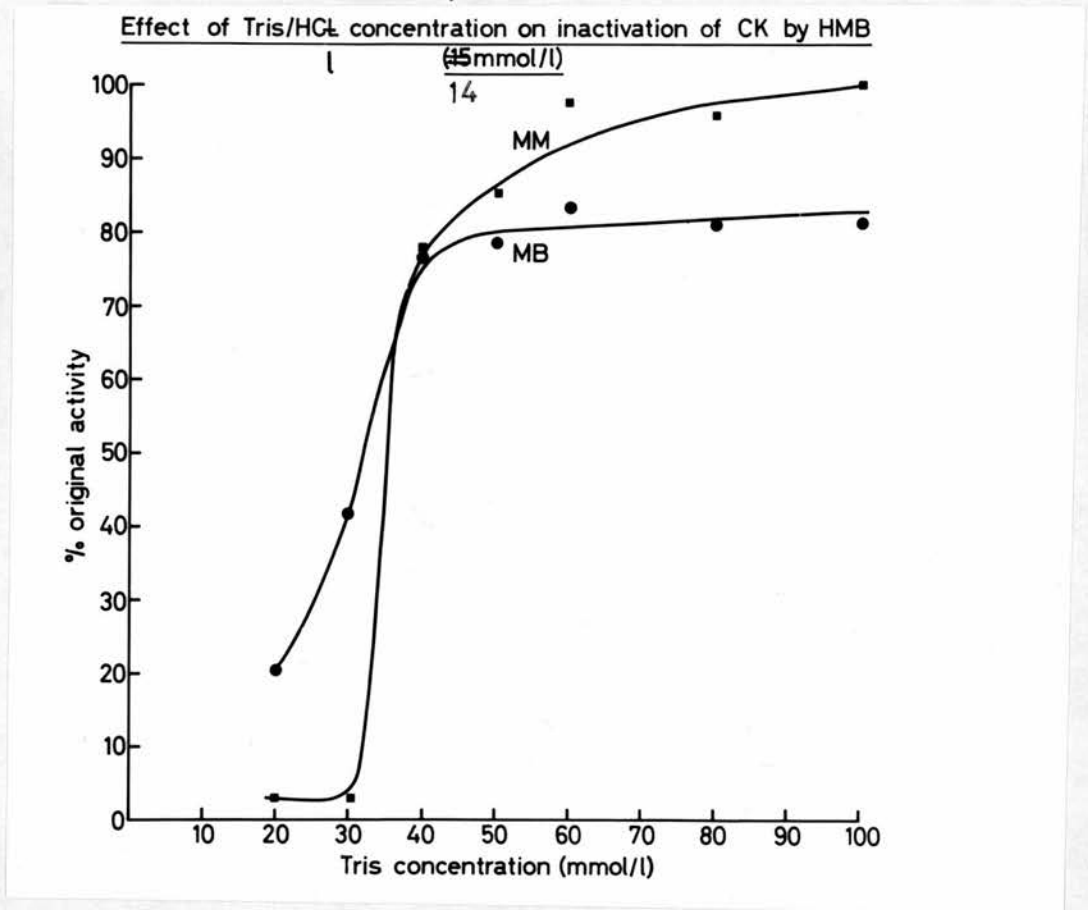




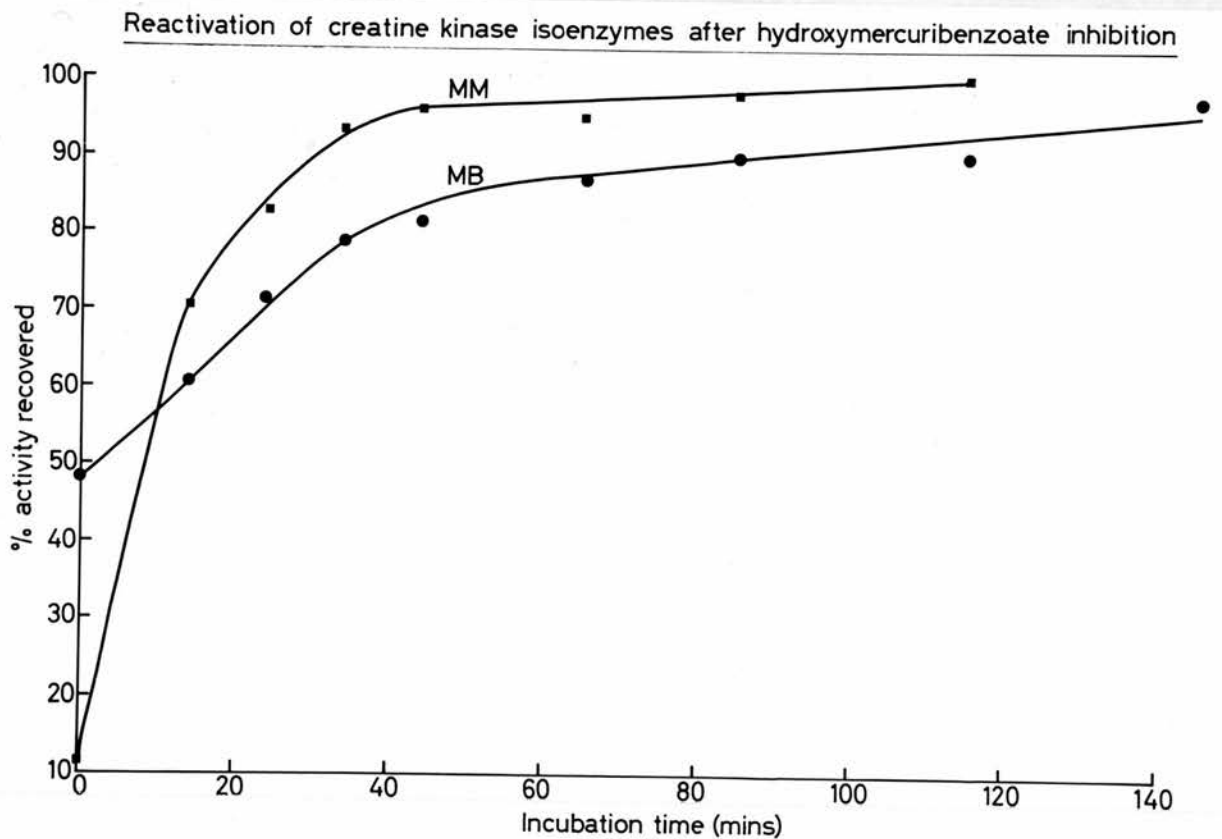
Figure 5-10

Inactivation of the MM and MB Isoenzymes of Creatine Kinase  
by p-hydroxymercuribenzoate

c.



d.



all at pH 8.0, in the presence of 14 mmol/l pHMB.

The rate of inactivation of both isoenzymes was found to vary with the nature of the buffer. In veronal buffer little inactivation of either isoenzyme was observed, but in phosphate and triethanolamine buffers both isoenzymes were inactivated (Table 5-5).

v) Effect of albumin. The total protein concentration in the incubation mixture was varied from 0-4 g/l, with 14 mmol/l pHMB in 30 mmol/l tris/HCl pH 8.0. Activity was determined after 90 min incubation at 2°- 3°C.

Bovine albumin had no significant protective effect on either isoenzyme up to 4 g/l.

vi) Reactivation in the assay solution. Since the assay solution contained 9 mmol/l GSH and the pHMB was diluted 44 times in it, reactivation of the isoenzymes in the assay solution was likely. It was studied by delivering aliquots of the incubation mixture after 90 min incubation into a series of assay solutions which were then incubated at 37°C. The activity was measured at regular intervals.

Both isoenzymes were rapidly reactivated in the assay solution. They recovered 65% of their original activity in about 15 min (Fig 5-10d).

### Discussion.

The inactivation and inhibition of enzymes by thiol-reacting reagents are known to be complex. The rate of inactivation depends on a large number of variables which include temperature, pH, purity of the enzyme sample,

Table 5-5

Effect of Various Buffers on Inactivation of Creatine Kinase Isoenzymes  
by Incubation for 90 min with p-hydroxymercuribenzoate (14 mmol/l) at

2° - 3° C

Isoenzyme	Original Activity IU/l	Tris/HCl IU/l	Phosphate IU/l	Triethanolamine IU/l	Veronal IU/l
MM	2500 (100%)	115 (4.6%)	102 (4.1%)	95 (3.8%)	2100 (84%)
MB	550 (100%)	230 (42%)	58 (11%)	58 (11%)	515 (94%)

concentrations of protein, enzyme and the thiol-reacting reagent, side reactions and the presence of protective reagents (e.g. enzyme substrates, cofactors and thiols). It is, therefore, unlikely that any simple conclusions can be drawn concerning the mode of action of the thiol-reacting reagents from these studies on the isoenzymes. Moreover, only partially purified enzymes were used. The value of the present results, therefore, is essentially comparative.

The more detailed study of the pHMB inactivation reveals the complexity of the above effects since the rate and extent of inactivation are influenced by the nature and concentration of the buffer as well as the concentration of pHMB. This, together with the high concentration of pHMB needed, makes it likely that side reactions other than with the enzyme thiol groups might have been involved. The reversible nature of the pHMB inactivation suggests that the mercaptide complex (if this is the complex formed) is not a tight one, and no permanent change in the enzyme site has occurred. The extent of the reactivation is determined by the duration and concentration of the thiol compounds in the enzyme assay solution.

Figure 5-9 and Table 5-4 show that a much higher concentration of pHMB than iodoacetamide was needed to produce the same degree of inhibition and inactivation, yet pHMB is known to be one of the most reactive thiol-reacting reagents. This might be due to the smaller size and the electric neutrality of the iodoacetamide molecule.

In the inhibition studies, 5 mmol/l  $\beta$ -mercaptoethanol

was present in the assay solution for the activation of the creatine kinase isoenzymes. It reacted with the thiol reagents and reduced their effective concentrations. So the observed concentration causing inhibition to half the original activity are suitable only for comparing the two isoenzymes.

### Conclusion.

In the same experimental conditions, the MM and MB isoenzymes behave similarly towards iodoacetamide and oxidised glutathione, but differently towards pHMB. Their greatest difference in inactivation by pHMB was achieved at 14 mmol/l pHMB in 30 mmol/l tris/HCl pH 8.0 after 60 - 70 min incubation at 2 - 3°C.

## 5-3 Enzymatic properties of the isoenzymes.

### 5-3-1 Effect of magnesium ions.

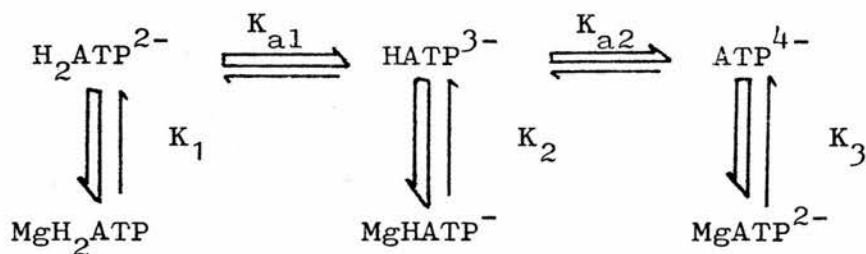
Many bivalent cations, for example  $Mg^{2+}$ ,  $Mn^{2+}$ ,  $Ca^{2+}$  and  $Co^{2+}$ , have been found to activate muscle creatine kinase (MM), while others like  $Ba^{2+}$ ,  $Sr^{2+}$ ,  $Ni^{2+}$ ,  $Cr^{2+}$ ,  $Cd^{2+}$  and  $Zn^{2+}$  are either inactive or inhibitory (O'Sullivan and Morrison, 1963). Only  $Mg^{2+}$  has been studied in this investigation.

Magnesium ions are the "natural" activators of the great majority of enzymes that act on phosphorylated

substrates, the kinases and synthetases. A plot of reaction velocity against concentration of the added cation is commonly a rectangular hyperbola similar to the curve of substrate concentration plotted against velocity, from which the " $K_m$ " of the cation can be obtained. The interpretation of such curves is not simple. The cation may activate by combining with the enzyme, with either of the substrates, or by a number of indirect means such as changing the equilibrium position of the reaction and removing inhibitors present in the reaction mixture. For creatine kinase it has been confirmed that  $Mg^{2+}$  activates by combining with the adenine nucleotide substrates. It has also been shown that only the ionic species  $MgADP^-$  and  $MgATP^{2-}$  are the real nucleotide substrates (Nihei et al., 1961; Noda et al., 1960).

The reaction of  $Mg^{2+}$  with the adenine nucleotides can be described by the following equilibria:

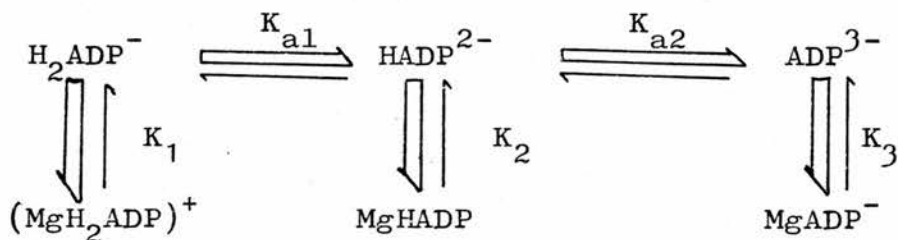
a. ATP



$$pK_{a1} = 4.0; \quad pK_{a2} = 6.9 \quad (\text{Melchion, 1954})$$

$$K_2 = 8000 \text{ mol}^{-1}; \quad K_3 = 90\,000 \text{ mol}^{-1} \quad (\text{O'Sullivan and Perry, 1964})$$

## 2. ADP



$$pK_{a1} = 3.9; \quad pK_{a2} = 6.65 \quad (\text{Melchion, 1954})$$

$$K_3 = 2000 \text{ mol}^{-1} \quad (\text{O'Sullivan and Perry, 1964})$$

$K_{an}$  = acid dissociation constants

$K_n$  = stability constants of  $\text{Mg}^{2+}$ -nucleotide complexes

From these equilibria it can be seen that the concentrations of the real substrates are dependent on 1) the relative magnitudes of the  $K_a$  and  $K_n$  values, 2) the amount of  $\text{Mg}^{2+}$  present and 3) the pH of the medium.

For ATP at pH 9.0 at which the forward reaction of creatine kinase was measured, the concentration of  $\text{ATP}^{4-}$  is  $10^{-6.9 + 9} = 10^{2.1}$  times greater than  $\text{HATP}^{3-}$ .  $\text{H}_2\text{ATP}$  is negligible at this pH. This ratio is made even smaller when  $\text{Mg}^{2+}$  is present in excess as  $K_3 \gg K_2$ . Under these conditions the existence of  $\text{MgHATP}^-$  and  $\text{HATP}^{3-}$  is negligible, and  $\text{MgATP}^{2-}$  concentration can be taken as that of the total ATP.

For ADP at pH 7.0 about 70 - 85% of total ADP exists as  $\text{ADP}^{3-}$ , and the rest as  $\text{HADP}^{2-}$ ,  $\text{H}_2\text{ADP}^-$  being negligible at this pH. The value of  $K_2$  at pH 7.0 is less well documented than  $K_3$ , but it is thought to be much smaller than  $K_3$  (Nihei *et al.*, (1961) neglected  $K_2$  in their

calculation of  $\text{MgADP}^-$  concentration). Thus, the equilibrium can still be made to shift overwhelmingly in favour of  $\text{MgADP}^-$  formation by adding sufficient  $\text{Mg}^{2+}$ , and in this circumstance the  $\text{MgADP}^-$  concentration approaches that of the total ADP.

The calculation of concentrations of  $\text{MgATP}^{2-}$  and  $\text{MgADP}^-$  from equilibrium constants is difficult because the accuracy of such calculations depends entirely on the precision of these constants. Unfortunately the values for the constants vary considerably with experimental conditions (O'Sullivan and Perry, 1964). Moreover, the kinetics of  $\text{Mg}^{2+}$  binding is further complicated by the facts that  $\text{Mg}^{2+}$  also, to a smaller extent, combines with  $\text{OH}^-$  and creatine phosphate (Morrison and James, 1965), and that  $\text{Na}^+$  and  $\text{K}^+$  with the adenine nucleotides (Melchion 1954).

#### Procedure.

Activity in the forward reaction was measured by the method of Tanzer and Gilvarg (1959), and the backward by the colorimetric method of Hughes (1962).

The effect of a range of  $\text{Mg}^{2+}$  concentrations (0-9 mmol/l), added as magnesium acetate, on the reaction velocity was studied at a fixed concentration of the nucleotide substrate. A family of curves was obtained at different concentrations of the nucleotide. The concentrations of creatine in the forward reaction and creatine phosphate in the backward reaction were kept constant throughout at 20 mmol/l and 10 mmol/l respectively.



The effect of  $Mg^{2+}$  on the activity of the backward reaction was also studied in the presence of varying concentrations of creatine phosphate with the ADP concentration kept at 2 mmol/l.

### Results.

In all studies similar results were obtained for the MM and MB isoenzymes.

All the  $Mg^{2+}$  concentration-velocity curves with varying concentrations of either nucleotide substrate (ATP or ADP) crossed each other, showing that at a fixed concentration of  $Mg^{2+}$  the reaction velocity was not directly proportional to the nucleotide concentration (Figs. 5-11a and b). This suggests that the  $Mg^{2+}$  binds with the nucleotide to form a real substrate complex rather than binding directly with the enzyme (Gutfreund, 1972). Inhibition by excess  $Mg^{2+}$  was evident in the forward reaction in the range of  $Mg^{2+}$  concentrations studied (Fig. 5-11a).

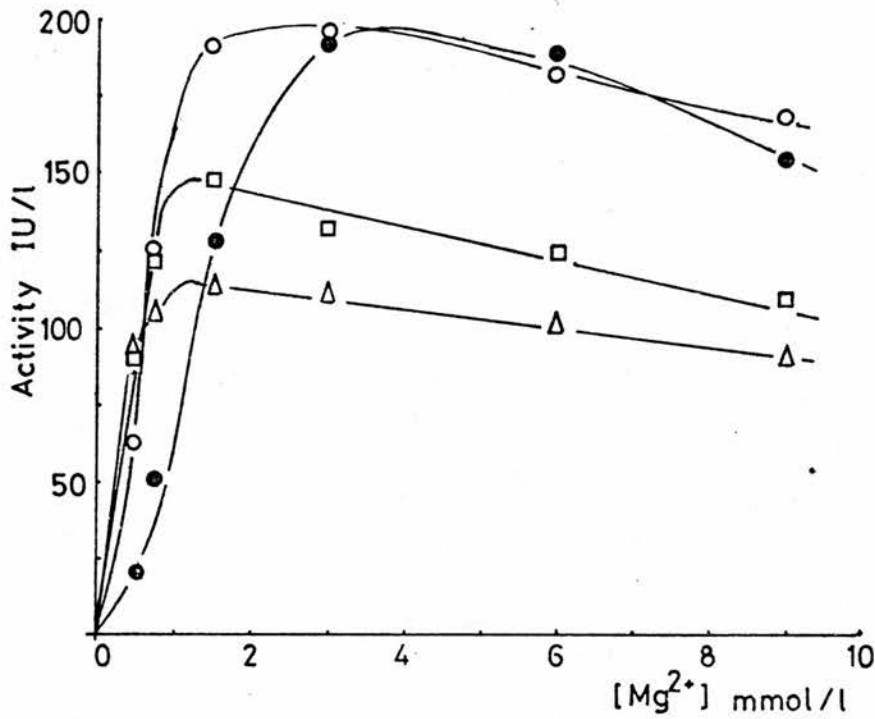
The  $Mg^{2+}$  concentration-velocity curves with varying concentrations of creatine phosphate did not, however, cross each other, suggesting that  $Mg^{2+}$  did not react with creatine phosphate to bind with the enzyme (Fig. 5-11c), although an inactive complex of  $Mg^{2+}$  with creatine phosphate has been shown to exist.

By replotting the velocity data with nucleotide substrates the optimal  $Mg^{2+}$ /nucleotide ratio was found to be 2 for the forward reaction and to lie between 5 and 10 for the backward reactions (Fig. 5-12a and b).

Figure 5-11

Effect of  $Mg^{2+}$  on the Activity of Creatine Kinase with Varying Concentrations of Adenine Nucleotide

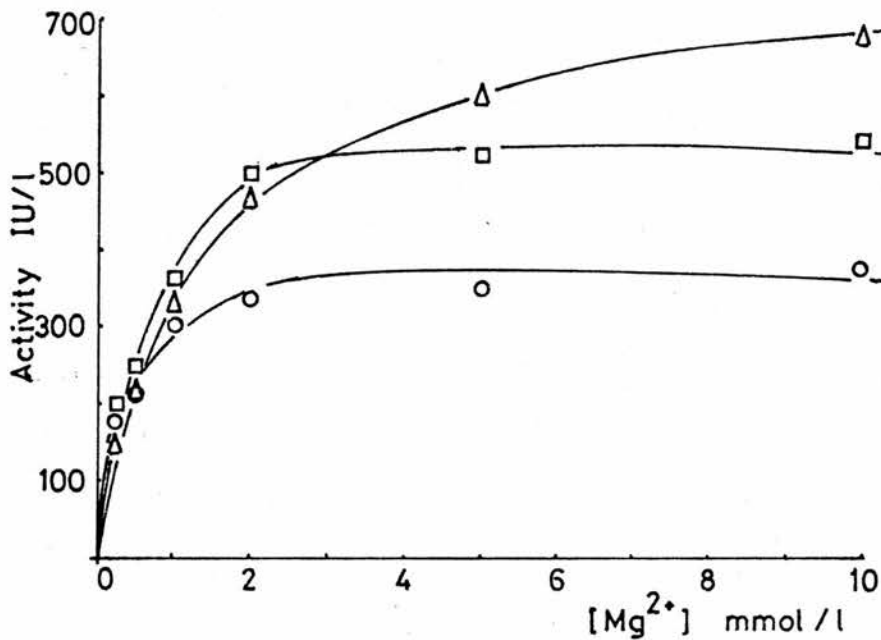
a. Forward Reaction with MB Isoenzyme



[Creatine] was kept constant at 20 mmol/l;

- Δ [ATP] 0.5 mmol/l;
- [ATP] 0.75 mmol/l;
- [ATP] 1.5 mmol/l;
- [ATP] 3.0 mmol/l.

b. Backward Reaction with MM Isoenzyme

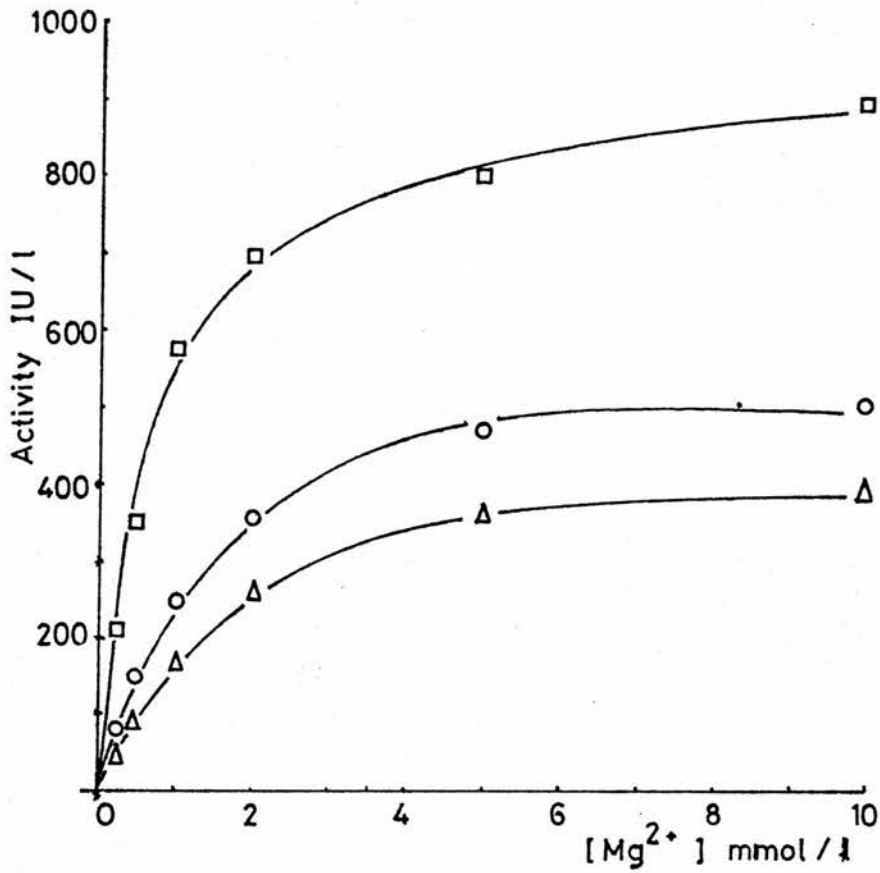


[Creatine phosphate] was kept constant at 10 mmol/l;

- [ADP] 0.5 mmol/l;
- [ADP] 1.0 mmol/l;
- Δ [ADP] 2.0 mmol/l.

Figure 5-11

c. Effect of  $Mg^{2+}$  on the Activity of Creatine Kinase with Varying Concentrations of Creatine Phosphate

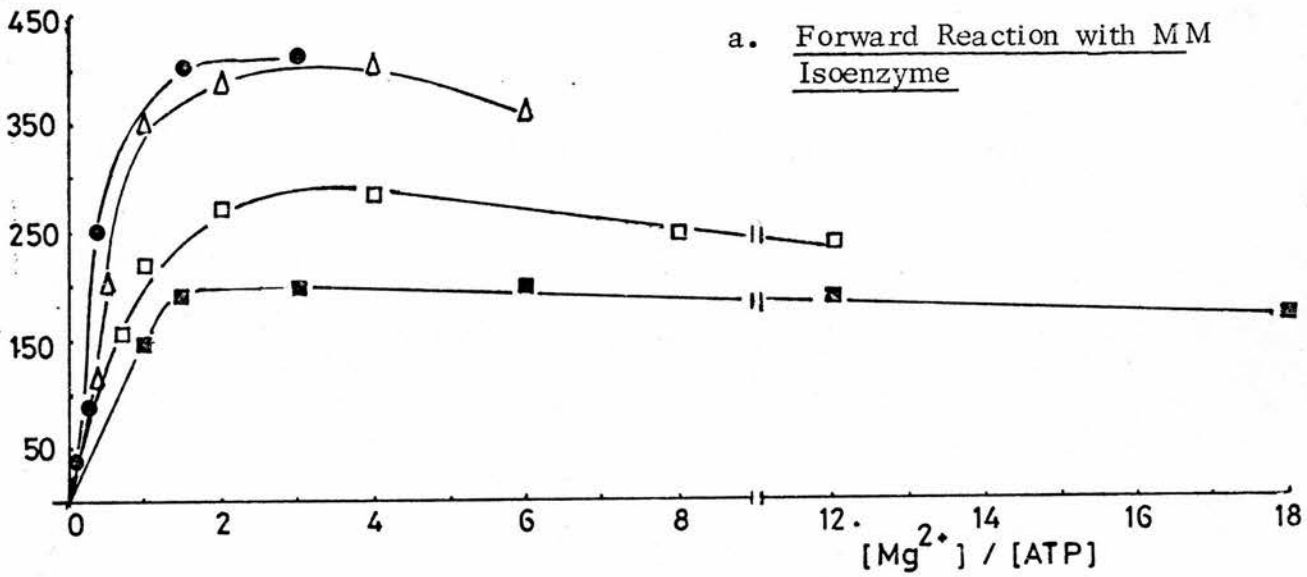


[ADP] was kept at 2 mmol/l;       $\Delta$  [Creatine phosphate] 1.25 mmol/l;  
 $\circ$  [Creatine phosphate] 2.50 mmol/l;       $\square$  [Creatine phosphate] 10 mmol/l.

Figure 5-12

Effect of  $[Mg^{2+}] / [Adenine\ Nucleotide]$  on the Activity of  
Creatine Kinase

Activity IU/l



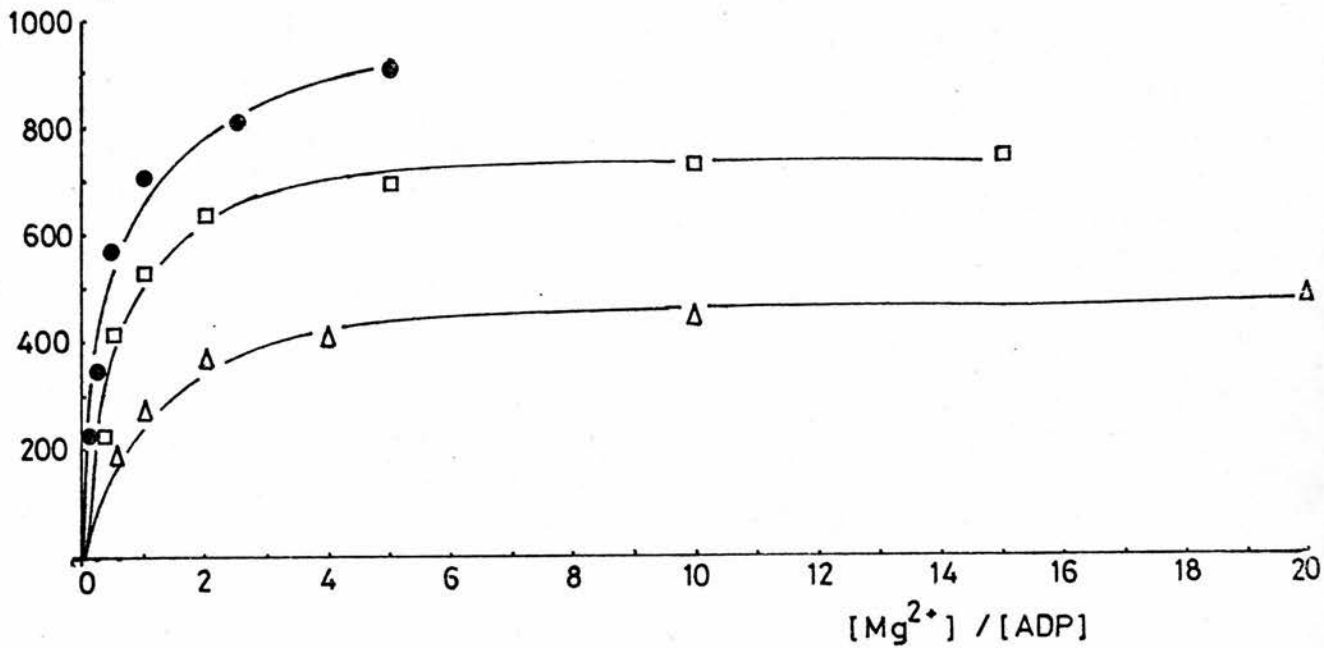
■ [ATP] 0.5 mmol/l;  
● [ATP] 3.0 mmol/l

□ [ATP] 0.75 mmol/l;

△ [ATP] 1.5 mmol/l

b. Backward Reaction with MB Isoenzyme

Activity IU/l



△ [ADP] 0.5 mmol/l;  
● [ADP] 2.0 mmol/l.

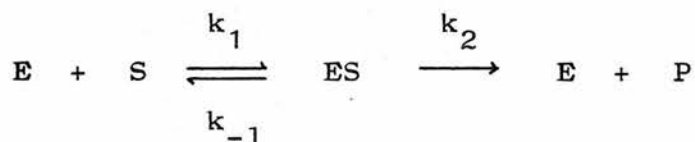
□ [ADP] 1.0 mmol/l;

Therefore, in all kinetic studies on creatine kinase described in this thesis,  $Mg^{2+}$  was present in the experimentally determined optimal  $Mg^{2+}$ /nucleotide ratio, and the assumption was made that the concentration of the  $Mg^{2+}$ -nucleotide substrates ( $MgATP^{2-}$  and  $MgADP^-$ ) equalled that of the total nucleotide.

### 5-3-2 Substrate affinities of the isoenzymes.

Based on an earlier suggestion of Henri (1902), Michaelis and Menton (1913) proposed that enzymic action could be explained by the formation of an enzyme-substrate complex which subsequently broke down into free enzyme and products. Their treatment was later generalised by Briggs and Haldane (1925) who assumed that the enzyme-substrate complex existed in a steady state during the reaction.

Single-substrate system. This system can be written as



According to the Steady-state theory,

$$v = V/(1 + K_m/S) \quad \text{.....1)}$$

where  $v$  = initial velocity of the reaction

$V$  = maximum velocity in a fixed concentration of enzyme

$P$  = products

$K_m$  = Michaelis constant

$k$  = rate constants

$S$  = substrate concentration

$E$  = enzyme concentration

The meaning of the  $K_m$  (which equals the substrate concentration at half maximum velocity) varies with the reaction mechanism and is represented by  $k_2 + k_1/k_{-1}$ .

To obtain the kinetic parameters, various ways of plotting the data derived from equation 1) can be used:-

1. v against S. A rectangular hyperbola is obtained where V can theoretically be estimated as the asymptotic value of v as S becomes very large (approaches infinity).  $K_m$  is obtained as the value of S at which  $v = V/2$ .

2. 1/v against 1/S (Lineweaver and Burk, 1934).

This double reciprocal plot is based on the linear equation

$$1/v = K_m/V \cdot \frac{1}{S} + 1/V$$

V and  $K_m$  can be worked out from the slope and intercept of the plot.

3. v against V/S (Eadie, 1942; Hofstee, 1952).

This is from the equation

$$v = V - K_m V/S$$

4. S/v against S (Hanes, 1932).

$$S/v = K_m/V + S/V$$

5. v against pS ( $-\log S$ ) (Dixon and Webb, 1964).

$$pS = pK_m + \log (V-v)/v$$

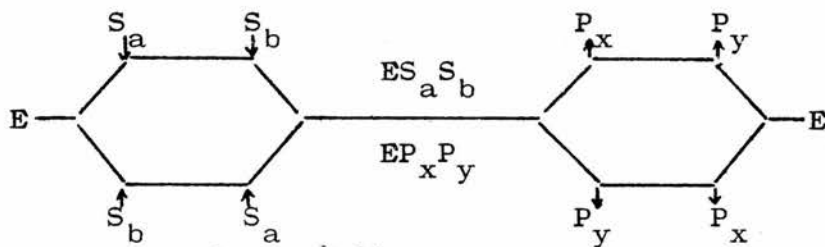
Because of their better precision, the linear plots (2, 3 and 4) are usually preferred to the non-linear ones (1 and 5). Of the three linear plots the Lineweaver-Burk method has been used most extensively although, when the line is drawn "by eye" or from simple "least squares" calculations,

it is the least satisfactory, since most points tend to group at one end of the line, if rate measurements are made at regular intervals of  $S$ , tending to cause imprecision in the estimation of  $V$  and  $K_m$ . The other two methods are better on this respect. The disadvantage of the Lineweaver-Burk plot can, however, be removed by using weighted non-linear regression analysis (Wilkinson, 1961). Cleland (1967) has provided computer programs for such statistical corrections.

Two-substrate systems. When two substrates are involved in an enzyme reaction the steady-state treatment does not necessarily give the "Michaelis-type" equation. There are, however, three conditions which give linear reciprocal plots in a two-substrate system.

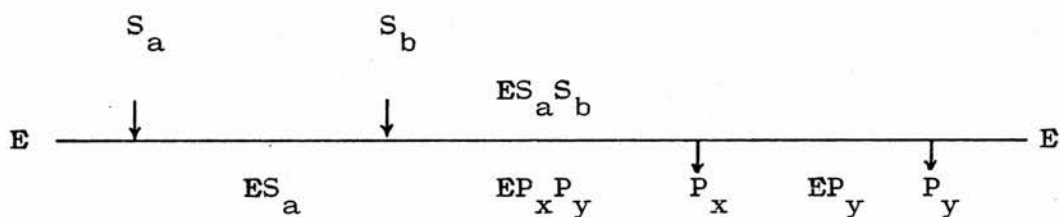
1. The rapid-equilibrium-random-order mechanism.

In this mechanism the enzyme ( $E$ ) can form a binary complex with either substrate ( $S_a$  or  $S_b$ ) and also the ternary complex,  $ES_aS_b$ , with no restriction to the order in which  $S_a$  and  $S_b$  become attached to the enzyme. The breakdown of the ternary complex into products ( $P_x$  and  $P_y$ ) is rate-determining and is so slow that it is in equilibrium with  $S_a$ ,  $S_b$ ,  $ES_a$  and  $ES_b$ . Using Cleland's shorthand notation the whole mechanism can be described as follows:



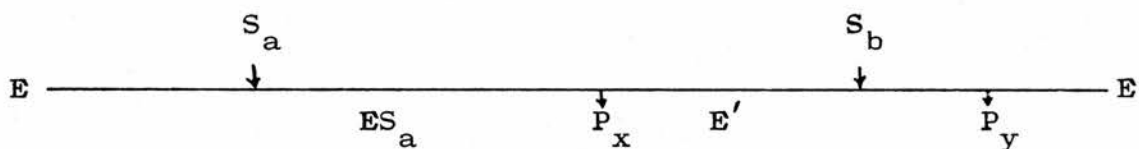
2. The ordered-ternary-complex mechanism.

In this mechanism one of the substrates ( $S_a$ ) combines with the enzyme so much more readily than the other substrate ( $S_b$ ) that  $ES_b$  hardly exists at all and the ternary complex  $ES_a S_b$  is formed virtually only from the  $ES_a$  complex. Cleland's notation for this mechanism is



3. The substituted-enzyme mechanism (ping-pong mechanism).

No ternary complex is formed in this mechanism. The enzyme reacts with one substrate to give one product and a substituted enzyme which further binds with the other substrate to yield another product and the free enzyme.



All these three mechanism yield the "Michaelis-type" rate equation. Using the usual notations,

$$V/v = 1 + K_a/S_a + K_b/S_b + K_{ab}/S_a S_b \dots\dots\dots 2)$$

$K_a, K_b$  = Michaelis constants for  $S_a$  and  $S_b$

$K_{ab}$  = constant whose meaning varies with the reaction mechanism.

The values of these constants can be obtained by plotting  $1/v$  against  $1/S_a$  at a constant value of  $S_b$ . A series of values of the  $1/v$  intercept are obtainable using



different values of  $S_b$ . These are plotted against  $S_b$ . The  $1/S_b$  intercept then gives  $1/K_b$ . The original plot of  $1/v$  against  $1/S_a$  for various values of  $S_b$  intersect at a point, giving  $-K_{ab}/K_a$ . A corresponding procedure of plotting  $1/v$  against  $1/S_b$  at various concentrations of  $S_a$  yields  $K_a$  and  $K_{ab}/K_b$ . (Florini and Vestling, 1957). Such study can give information concerning the reaction mechanism. If  $K_{ab}$  is zero, as in the substituted enzyme mechanism, a series of parallel lines will be obtained in the double reciprocal plot. If  $K_{ab}$  is not zero, the lines in the double reciprocal plot converge to a point, suggesting the presence of a ternary complex. Unfortunately, such plots cannot distinguish the random from the ordered ternary complex mechanisms. Other investigations, such as product inhibition studies, are needed to discriminate between these two mechanisms.

#### Procedure and Results.

The substrate affinities of the isoenzymes were studied in both the forward and backward reactions.

In the forward reaction, initial velocities were measured at pH 9.0 at  $37^\circ\text{C}$  by the method of Tanzer and Gilvarg (1959). The range of substrate concentrations was i) creatine 2.5 mmol/l to 20 mmol/l, ii) ATP 0.25 mmol/l to 3.0 mmol/l with  $\text{Mg}^{2+}/\text{ATP}$  ratio kept constant at 2.

In the backward reaction, the method of Hughes (1962) was used to measure initial velocity at pH 7.0 at  $37^\circ\text{C}$ . The range of substrate concentrations was i) creatine

phosphate 0.33 mmol/l to 10 mmol/l, ii) ADP 25  $\mu$ mol/l to 750  $\mu$ mol/l. The  $Mg^{2+}$ /ADP ratio was held at 10 throughout.

The results were analysed by the double reciprocal plot using the method of Florini and Vestling as described above. A computer program (Wilkinson, 1961; Hoy and Goldberg, 1971) was used to help analyse the data.

Some of the reciprocal plots are shown in Figs. 5-13a and b; 5-14a, b, c and d.

For both isoenzymes, in both forward and backward reactions, the lines in the primary plots intersect giving the constants  $K_{ab}/K_b$  and  $K_{ab}/K_a$ . The Michaelis constants for various substrates were derived from the secondary plots.

The values of all these kinetic parameters are listed in Table 5-6.

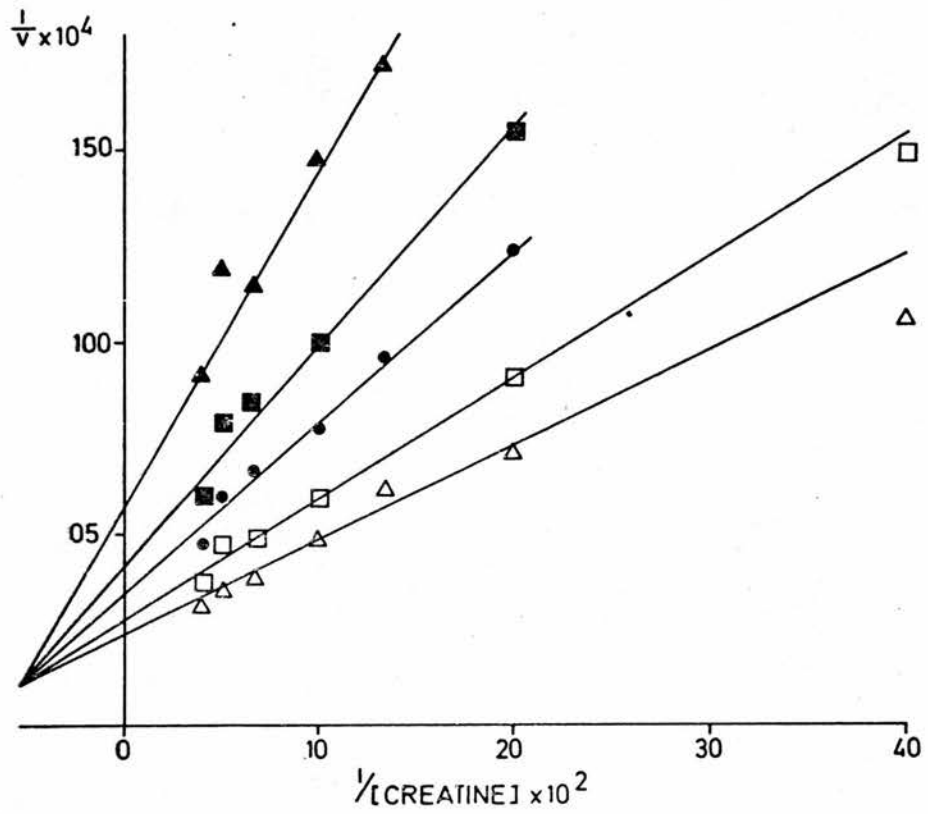
### Discussion.

The present results suggest the presence of enzyme-substrate ternary complexes for both the MM and MB isoenzymes. It has already been confirmed by many investigators (Morrison and James, 1965; Jacobs and Kuby, 1970) for the MM and BB isoenzymes that the reaction is of the random-rapid-equilibrium type. Since MB isoenzyme is a hybrid of the MM and BB isoenzymes, it seems reasonable to propose the same reaction mechanism for the MB isoenzyme. (This is further supported by the product inhibition study described in a later subsection). On this assumption, the kinetic parameters obtained will have the physical

Figure 5-13

Double Reciprocal Plot for the Creatine Kinase Isoenzymes

- a. Forward Reaction: MB Isoenzyme with Variation in Creatine Concentration at Different ATP Concentrations



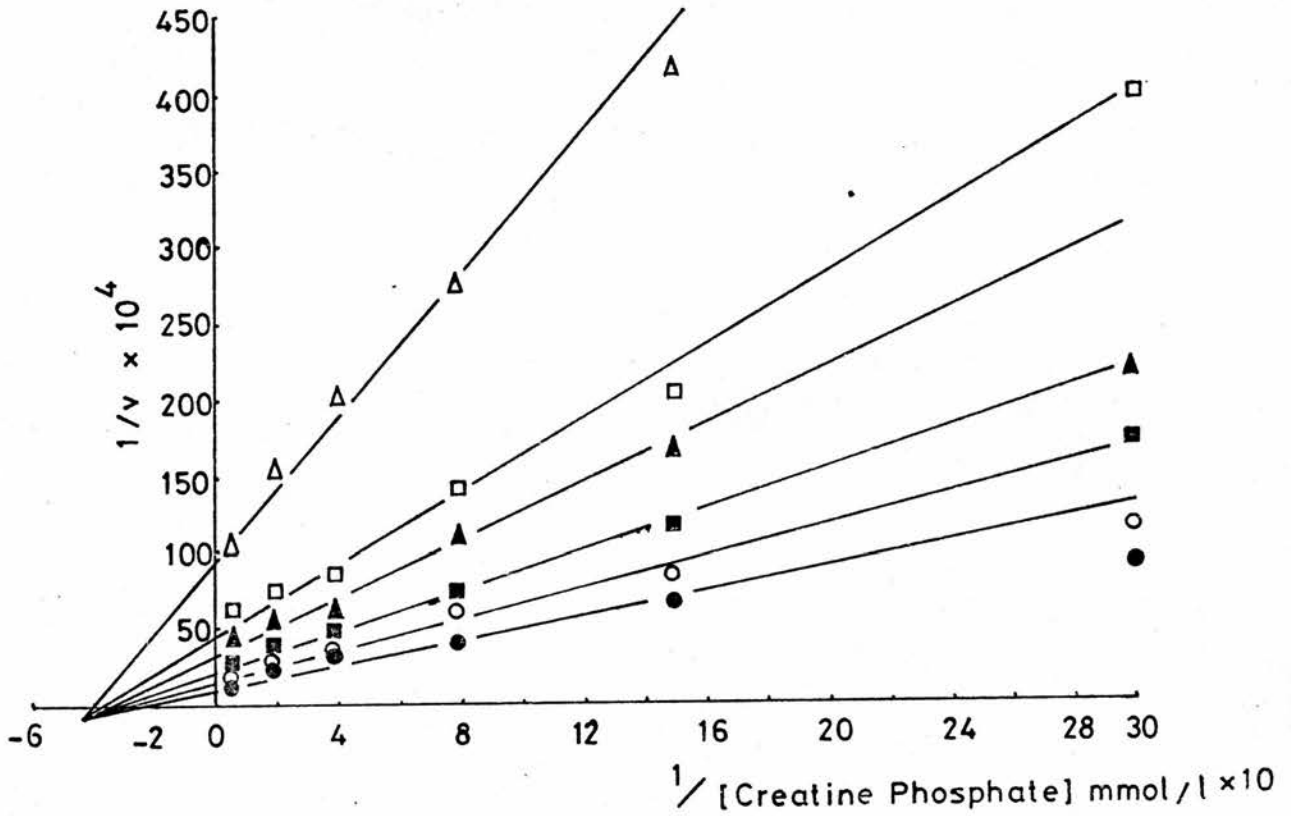
[ATP]:

▲ 3.0 mmol/l;    ■ 1.5 mmol/l;    ● 1.0 mmol/l;    □ 0.75 mmol/l;  
△ 0.5 mmol/l.

Figure 5-13

Double Reciprocal Plot for the Creatine Kinase Isoenzymes

b. Backward Reaction: MM Isoenzyme with Variation in Creatine Phosphate Concentration at Different ADP Concentrations



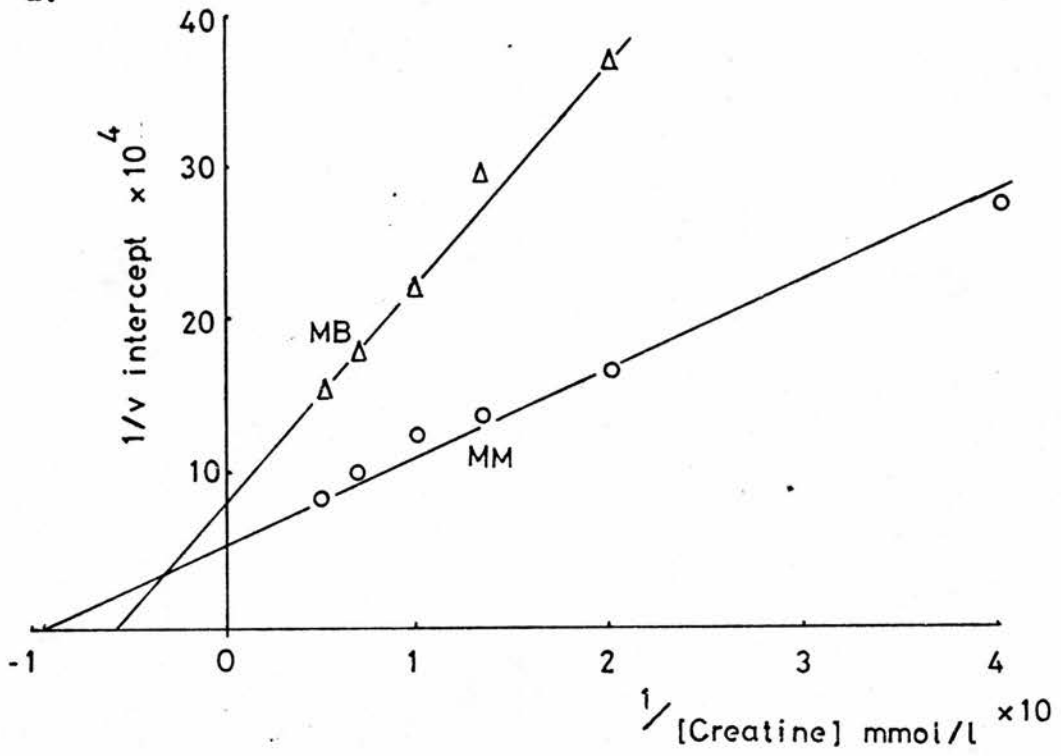
[ADP]:

- |                            |                           |                           |                            |
|----------------------------|---------------------------|---------------------------|----------------------------|
| ● 500 $\mu\text{mol/l}$ ;  | ○ 250 $\mu\text{mol/l}$ ; | ■ 125 $\mu\text{mol/l}$ ; | ▲ 87.5 $\mu\text{mol/l}$ ; |
| ◻ 62.5 $\mu\text{mol/l}$ ; | △ 25 $\mu\text{mol/l}$ .  |                           |                            |

Figure 5-14

Secondary Plots for the MM and MB Isoenzymes  
Forward Reaction

a.



b.

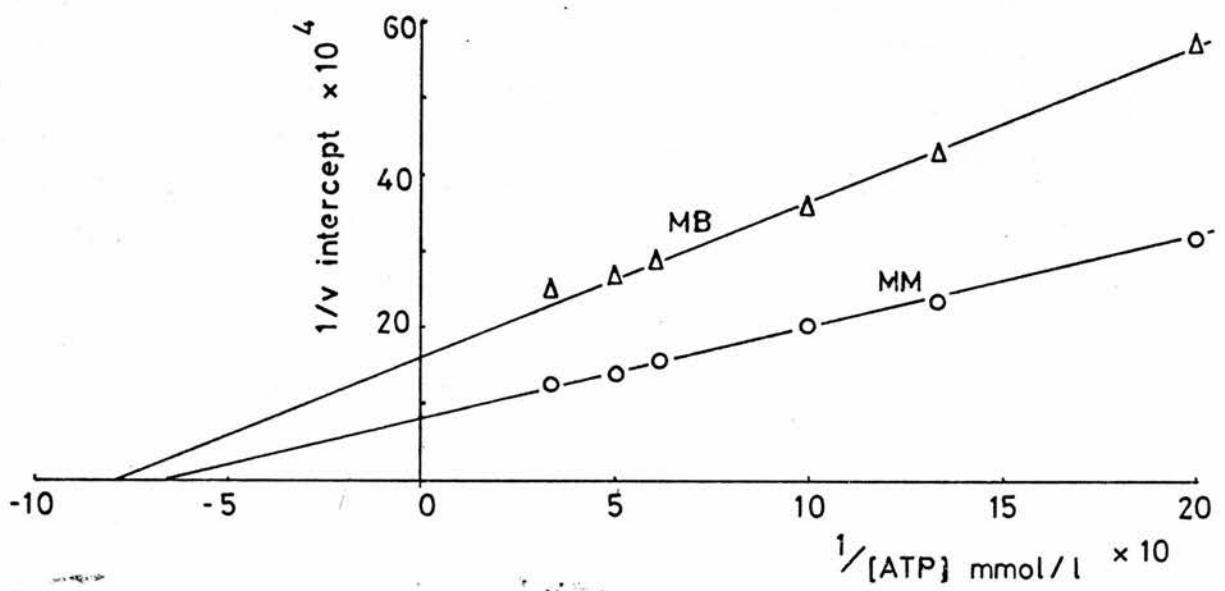
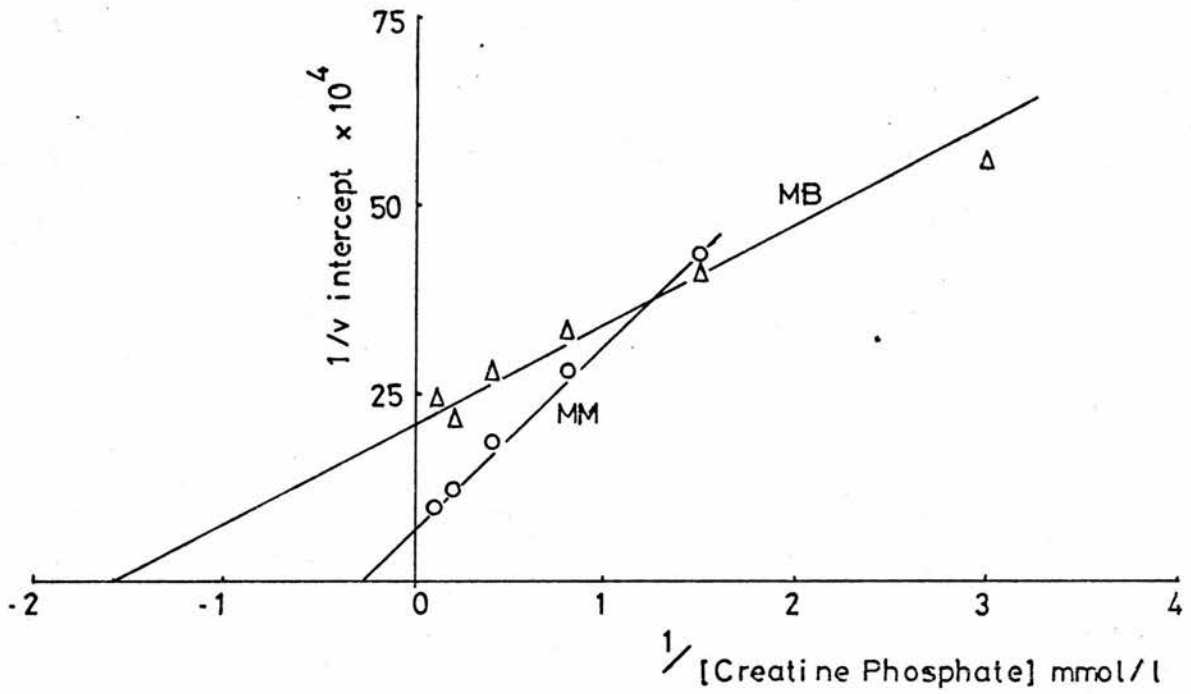


Figure 5-14

Secondary Plots for the MM and MB Isoenzymes

Backward Reaction

c.



d.

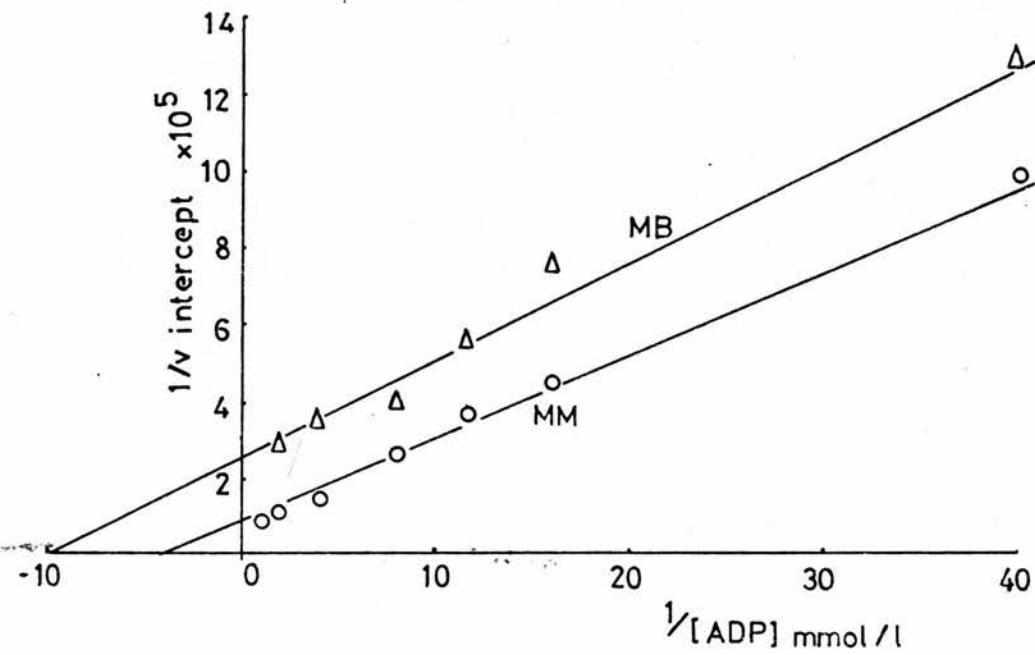
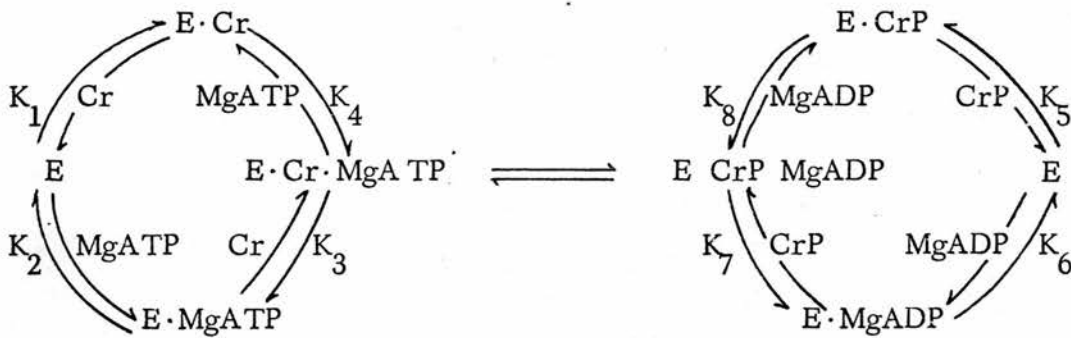


Table 5-6

Enzyme Substrate Affinities for the MM and MB Isoenzymes of  
Creatine Kinase



Kinetic Constant	Dissociation Constant	Equilibrium Forward Reaction (pH 9)	MB Isoenzyme	MM Isoenzyme
$K_{ab}/K_b$	$K_1$	$E \cdot Cr \rightleftharpoons E + Cr$	19.2 mmol/l	14.2 mmol/l
$K_{ab}/K_a$	$K_2$	$E \cdot MgATP \rightleftharpoons E + MgATP$	1.75 mmol/l	2.8 mmol/l
$K_a$	$K_3$	$E \cdot MgATP \cdot Cr \rightleftharpoons E \cdot MgATP + Cr$	20.6 mmol/l (S.D. = 3.6)	10.0 mmol/l (S.D. = 2.2)
$K_b$	$K_4$	$E \cdot MgATP \cdot Cr \rightleftharpoons E \cdot Cr + MgATP$	1.27 mmol/l (S.D. = 0.14)	1.43 mmol/l (S.D. = 0.17)
<u>Backward Reaction (pH 7)</u>				
$K_{cd}/K_d$	$K_5$	$E \cdot CrP \rightleftharpoons E + CrP$	0.77 mmol/l	2.08 mmol/l
$K_{cd}/K_c$	$K_6$	$E \cdot MgADP \rightleftharpoons E + MgADP$	0.11 mmol/l	0.24 mmol/l
$K_c$	$K_7$	$E \cdot MgADP \cdot CrP \rightleftharpoons E \cdot MgADP + CrP$	0.59 mmol/l (S.D. = 0.1)	3.54 mmol/l (S.D. = 0.2)
$K_d$	$K_8$	$E \cdot MgADP \cdot CrP \rightleftharpoons E \cdot CrP + MgADP$	0.11 mmol/l (S.D. = 0.02)	0.29 mmol/l (S.D. = 0.02)

$K_a$  = Michaelis constant for creatine (Cr)

$K_b$  = Michaelis constant for MgATP

$K_c$  = Michaelis constant for creatine phosphate (CrP)

$K_d$  = Michaelis constant for MgADP

$K_{ab}, K_{cd}$  = Kinetic constants

meanings described in Table 5-6.

It has been assumed that the effective concentrations of  $Mg^{2+}$ -nucleotide complexes approach those of the total nucleotides (as described in the last sub section). So far as this is valid, the kinetic parameters calculated for the total nucleotide may be taken as good approximations of those of the corresponding  $Mg^{2+}$ -nucleotide substrates.

### 5-3-3

#### Effect of pH.

pH may affect enzyme activity by i) irreversibly inactivating the enzyme at extreme pH values, ii) altering the reaction rate ( $V_{max}$ ) and iii) changing the enzyme substrates affinities ( $K_s$ ). The second and third effects are usually reversible.

The effect of irreversible inactivation can be studied by exposing the enzyme to a range of pH values and then assaying the activity at a pH at which the enzyme is stable. This effect can be distinguished from those on  $K_s$  and  $V_{max}$  by comparing such a pH-stability curve with the pH-activity curve (enzyme activity being determined at various pH values) which shows the overall effect of pH.

In order to investigate the effects of varying pH upon  $V_{max}$  and  $K_s$  independently of each other, the kinetics of the ionisation of the enzyme, substrates and enzyme-substrate complexes have been studied (Michaelis and Davidsohn, 1911; von Euler et al., 1924; Dixon, 1953;



Laidler, 1955). The following is a summary of the findings:

pH affects the enzyme activity by changing the ionic forms of enzymes (E), substrates (S) and enzyme-substrate complexes (ES).

The effective concentrations of E, S and ES at any particular pH are inversely proportional to their respective "pH functions" (f),  $f_E$ ,  $f_S$  and  $f_{ES}$ . Hence

$$V = k(ES) = \bar{k}(ES)/f_{ES} \dots\dots\dots 1)$$

$$\begin{aligned} K_s &= E \cdot S / (ES) = \frac{E^* S^*}{(ES)^*} \times \frac{f_{ES}}{f_E f_S} \\ &= \bar{K}_s \frac{f_{ES}}{f_E f_S} \dots\dots\dots 2) \end{aligned}$$

It can also be proved that in steady state kinetics

$$K_m = \bar{K}_m \frac{f_{ES}}{f_E f_S} \dots\dots\dots 3)$$

$\bar{k}$  = that part of the rate constant, k, which is independent of pH

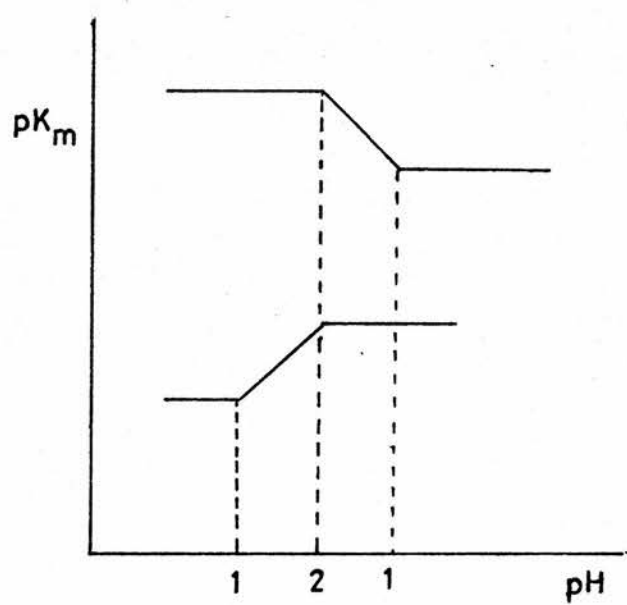
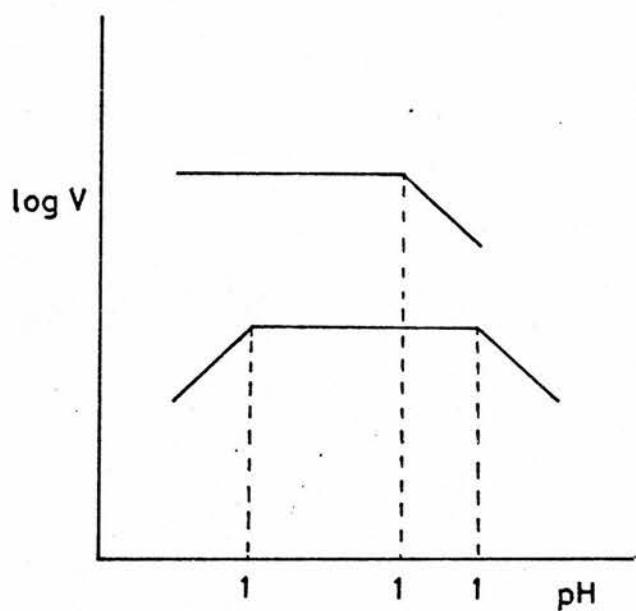
$\bar{K}_s$  = that part of the dissociation constant of substrate,  $K_s$ , independent of pH

$\bar{K}_m$  = that part of Michaelis constant,  $K_m$ , independent of pH

\* = active ionic form

V = maximum velocity at a given concentration of enzyme

A plot of negative log f (pf) against pH yeilds straight lines of 0, 1 or 2 unit slopes, giving the negative logarithm



1 =  $\text{pK}$  for ES      2 =  $\text{pK}$  for E or S

of the dissociation constants ( $pK$ ) of various ionic forms at the intersections.

A plot of  $\log V$  against  $pH$  is equivalent to plotting  $pf_{ES}$  against  $pH$  and hence it gives the  $pK$  values of the ES complex (Equation 1). Similarly, plots of  $pK_m$  against  $pH$  are equivalent to plotting  $(pf_E + pf_S - pf_{ES})$  against  $pH$  and hence yielding  $pK$  values for E, S and ES (Equation 3). Therefore, with this method of plotting the dissociation constants of the chemical groups essential for activity in E, S and ES can be found.

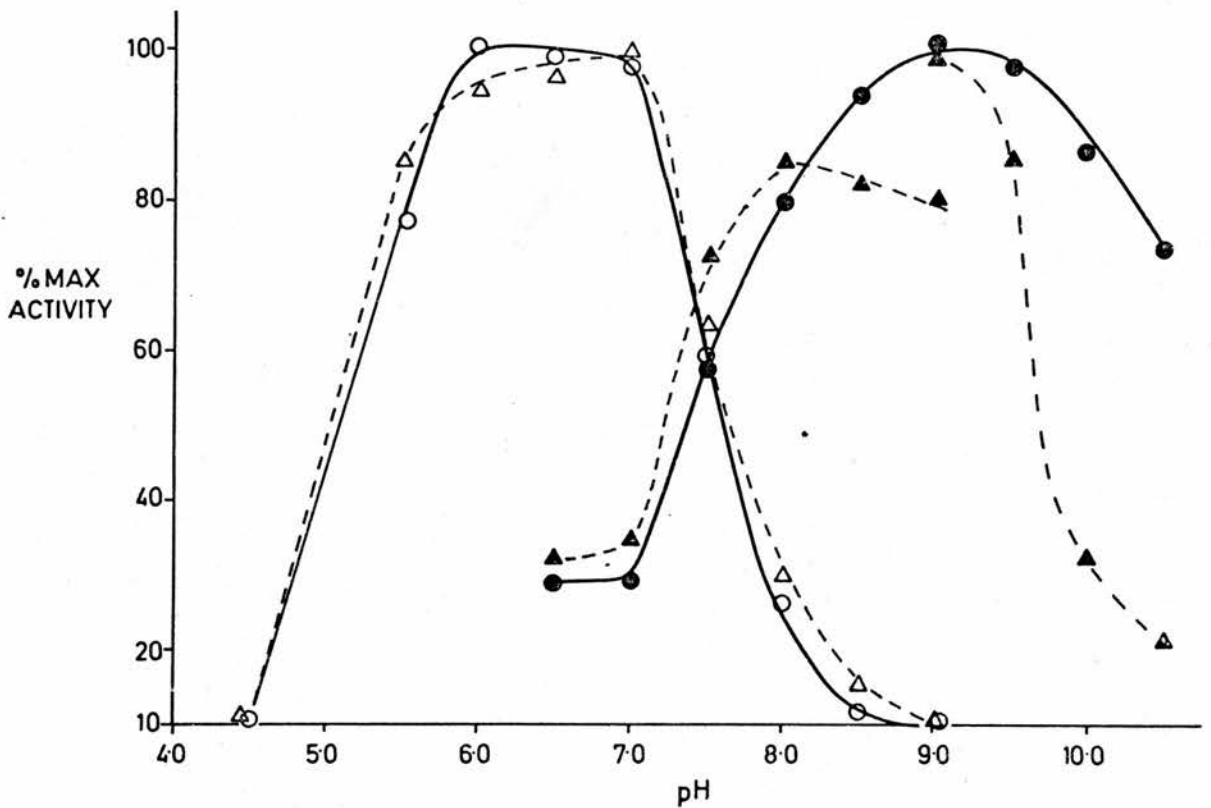
#### Procedure and Results.

1. The overall effect of  $pH$  on enzyme activity was studied by measuring the activity of the two isoenzymes at various  $pH$  values ranging from 4.5 to 10.5. The forward reaction was measured by the method of Tanzer and Gilvarg (1959) and the backward reaction by the method of Hughes (1962). The buffers used were i) 50 mmol/l sodium succinate  $pH$  4.5 - 6.5, ii) 50 mmol/l tris/HCl  $pH$  6.5 - 9.0 and iii) 50 mmol/l glycine/NaOH  $pH$  9.0 - 10.5.

In the forward reaction the two isoenzymes had slightly different  $pH$  optima with the substrate concentrations used,  $pH$  9.0 for the MM isoenzyme and  $pH$  8.0 - 9.0 for the MB. The MB isoenzyme had a higher activity (about 20%) in glycine buffer than in tris but the MM isoenzyme did not show this difference. In the backward reaction both isoenzymes showed peak activities at  $pH$  6.5 - 7.0 (Fig. 5-15).

Figure 5-15

pH -activity Curves for Creatine Kinase Isoenzymes



- Backward reaction MM isoenzyme;
- △---△ Backward reaction MB isoenzyme;
- Forward reaction MM isoenzyme;
- ▲---▲ Forward reaction MB isoenzyme.

Buffers used were:

- 50 mmol/l succinate buffer pH 4.5-6.5;
- 50 mmol/l tris/HCl buffer pH 6.5-9.0;
- 50 mmol/l glycine/NaOH pH 9.0-10.5.

2. The stability of the isoenzymes in the pH range 4.5 - 10.5 was studied by incubating the isoenzymes at 37°C for 15 min in 50 mmol/l buffers at different pH values, the buffer types used being the same as in the previous experiment. The protein concentration of the incubation mixtures for both isoenzymes was adjusted to 0.8 g/l with pure bovine albumin. At the end of the incubation 50  $\mu$ l portions were removed and assayed immediately by the method of Rosalki (1967) using an automated reagent kit (Boehringer, England).

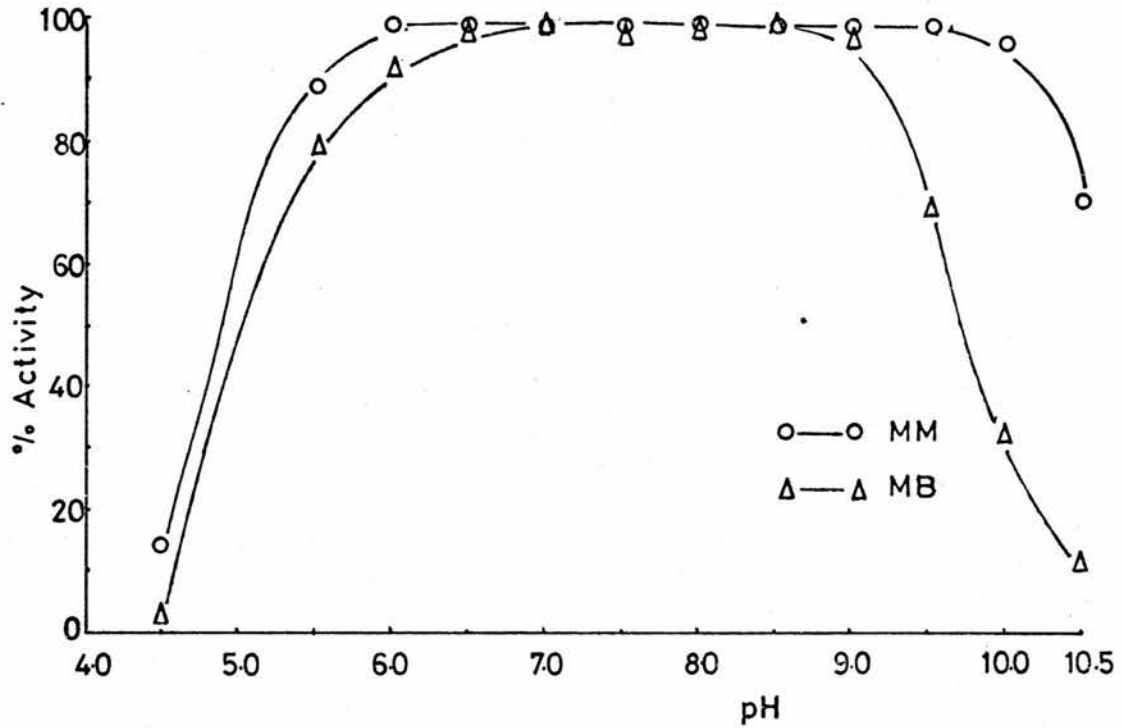
The MB isoenzyme was found to be less stable to extreme pH than the MM isoenzyme having a stable range of 6.4 - 9.0 pH units as opposed to pH 6.0 - 10.0 for the MM isoenzyme (Fig. 5-16).

3. Since the  $K_m$  values in the backward reaction differ widely between the isoenzymes, the pH effect on  $V$  and  $K_m$  in this reaction was selected for study. The Rosalki (1967) method was used to measure the initial velocity. The  $V$  and  $K_m$  values were obtained at different pH values from 6.2 - 8.2 with the same procedure as before (5-3-2). The  $pK_m$  and  $\log V$  values were plotted against pH (Dixon 1953).

Over the range pH 6.5 - 7.5, the  $K_m$  of the MB isoenzyme for  $MgADP^-$  decreased by a factor of two, while that of the MM increased two fold. In the same range, the  $K_m$  of the MB isoenzyme for creatine showed little change while that of the MM isoenzyme showed a three fold increase. The change of  $V$  was similar for both isoenzymes. It

Figure 5-16

Stability of the Activity of Creatine Kinase Isoenzymes at  
Different pH Values



The isoenzymes were incubated in buffers at different pH values ( Succinate pH 4.5-6.5; tris/HCl pH 6.5-9.0; glycine/NaOH pH 9.0-10.5 all at 50 mmol/l) for 15 min at 37°C. Their activities were measured by the Rosalki method (1967) at pH 7.0 using 50  $\mu$ l aliquots of the incubation solution.

showed little change from pH 6.2 - 7.0, but there was a sharp drop at pH values above 7.0.

When the results were plotted according to the method of Dixon (1953), all the plots, except that for the  $pK_m$  for creatine phosphate for the MM isoenzyme and  $\log V$  for the MB isoenzyme, could be fitted into straight lines of zero and unit slopes, showing intersections at the following pH values (Fig. 5-17a and b):

	MM Isoenzyme		MB Isoenzyme	
Change of slope	+ 1	- 1	+ 1	- 1
Log V	-	pH 7.4	-	-
$pK_m \text{ MgADP}^-$	pH 7.4	pH 7.0	pH 6.9	pH 7.2
$pK_m$ creatine phosphate	-	-	-	pH 7.55

#### Discussion.

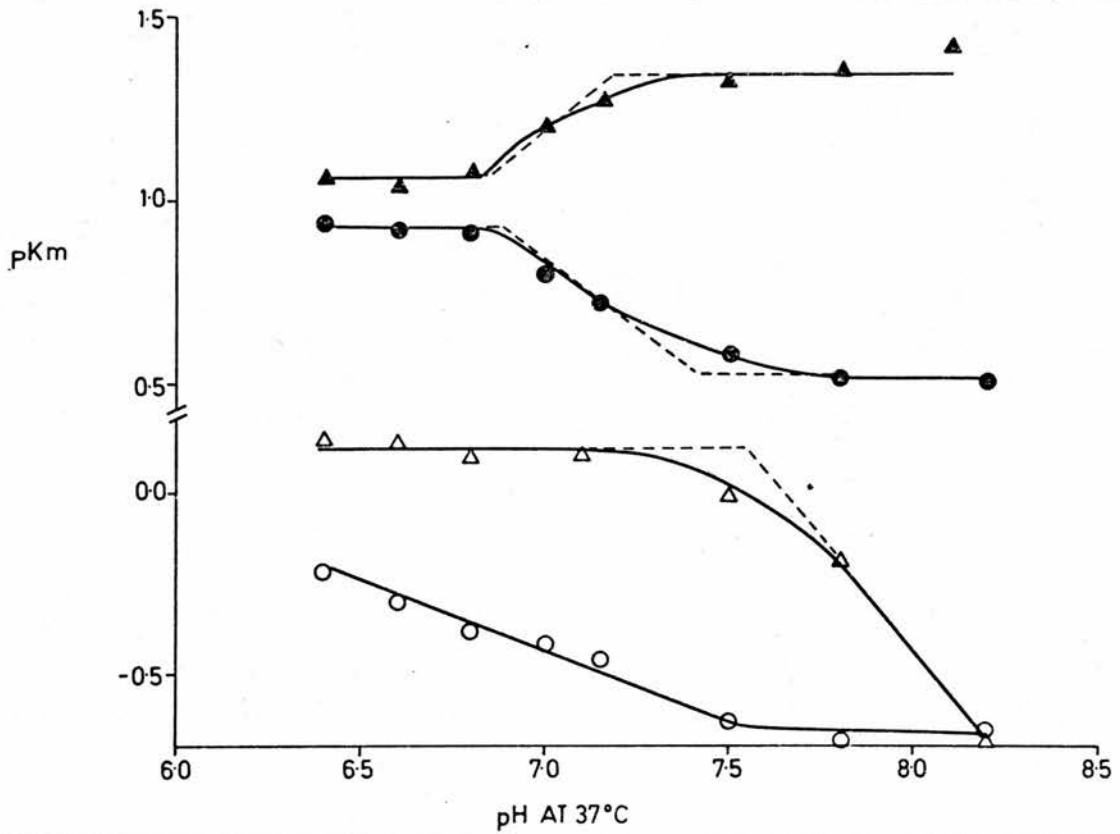
The two isoenzymes have been shown under the experimental conditions used in this investigation to have similar pH optima in both forward and backward reactions. These values, however, may change with substrate concentration and temperature. Similarly, the pH stability profiles are also not absolute, being dependent on incubation temperature, incubation time and exact composition of the incubation mixture.

It can be seen from the results of 3 above that the greatest difference between the two isoenzymes in the  $K_m$  values for both  $\text{MgADP}^-$  and creatine phosphate are found at pH 7.5. This may be important for the selective analysis

Figure 5-17

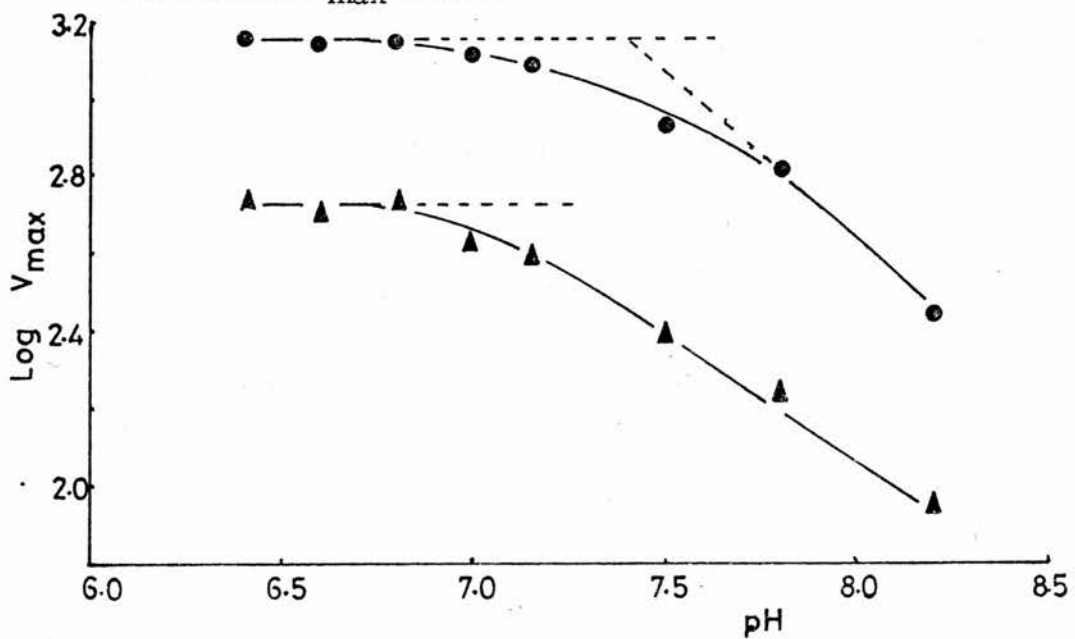
Variation of Kinetic Parameters with pH for the MM and MB Isoenzymes for the Backward Reaction on

a. Variation of  $pK_m$  with pH



$\blacktriangle$  MgADP MB isoenzyme;  $\bullet$  MgADP MM isoenzyme;  
 $\triangle$  creatine phosphate MB isoenzyme;  $\circ$  creatine phosphate MM isoenzyme.

b. Variation of  $V_{max}$  with pH



$\bullet$  MM isoenzyme;  $\blacktriangle$  MB isoenzyme.



of the MB isoenzyme. However, the sensitivity of the assay, for both isoenzymes, is less at pH 7.5 than pH 7.0, as  $V$  is much lower at pH 7.5 than at pH 7.0.

It is difficult to interpret the changes of slope in the  $\log V$  and  $pK_m$  plots especially in the two-substrate-ternary-complex system postulated for creatine kinase. According to the rules of Dixon (1953), the change at pH 7.4 may be attributable to the  $pK$  value of the enzyme-substrate complex (probably the ternary complex) of the MM isoenzyme; likewise the change at pH 6.9 may be attributable to a  $pK_{ES}$  of the MB isoenzyme. The changes in slope at pH 7.2 and pH 7.55 for the MB isoenzyme and at pH 7.0 for the MM might be ascribed to the  $pK$  values of free substrates, free enzyme or, in a two-substrate system, the binary complex between enzyme and complementary substrate. Since the two dissociation constants of creatine phosphate are  $pK_1 = 2.7$  and  $pK_2 = 4.58$ , it seems unlikely that these changes of slope refer to its ionisation. However, MgADP, which has a  $pK$  value of 6.6, might be responsible for the change at pH 7.0 for the MM isoenzyme. The assignment of these apparent  $pK$  values to a particular ionising group is, however, very hazardous as the dissociation of the ionising groups in free and protein bound forms can be very different. Moreover, the whole procedure will be invalidated if changes in pH cause a change in the rate-determining step of the enzyme reaction.

5-3-4

Effect of ionic strength.

This was studied by measuring the activities of the isoenzymes in the forward reaction (Tanzer and Gilvarg, 1959) in the presence of increasing glycine/NaOH buffer concentration and increasing concentration of NaCl.

It was found that while both isoenzymes were inhibited at high NaCl concentrations, only the MB isoenzyme was progressively inhibited by increasing strength of glycine/NaOH buffer (Fig. 5-18).

It is not certain how much the inhibitory effect at high concentrations of NaCl is due to increase of ionic strength and how much to specific inhibition by  $\text{Cl}^-$  (see 5-3-7).

5-3-5

Temperature effect.

This subsection deals with the effect of assay temperature on reaction velocity. Heat inactivation of the isoenzymes has already been discussed in 5-2-3.

In most chemical reactions, an increase in temperature is accompanied by an increase in reaction rate, as is described in the Arrhenius equation,

$$2.303 \log k = -E_a R \frac{1}{T} + C$$

k = kinetic rate constant

$E_a$  = Arrhenius energy

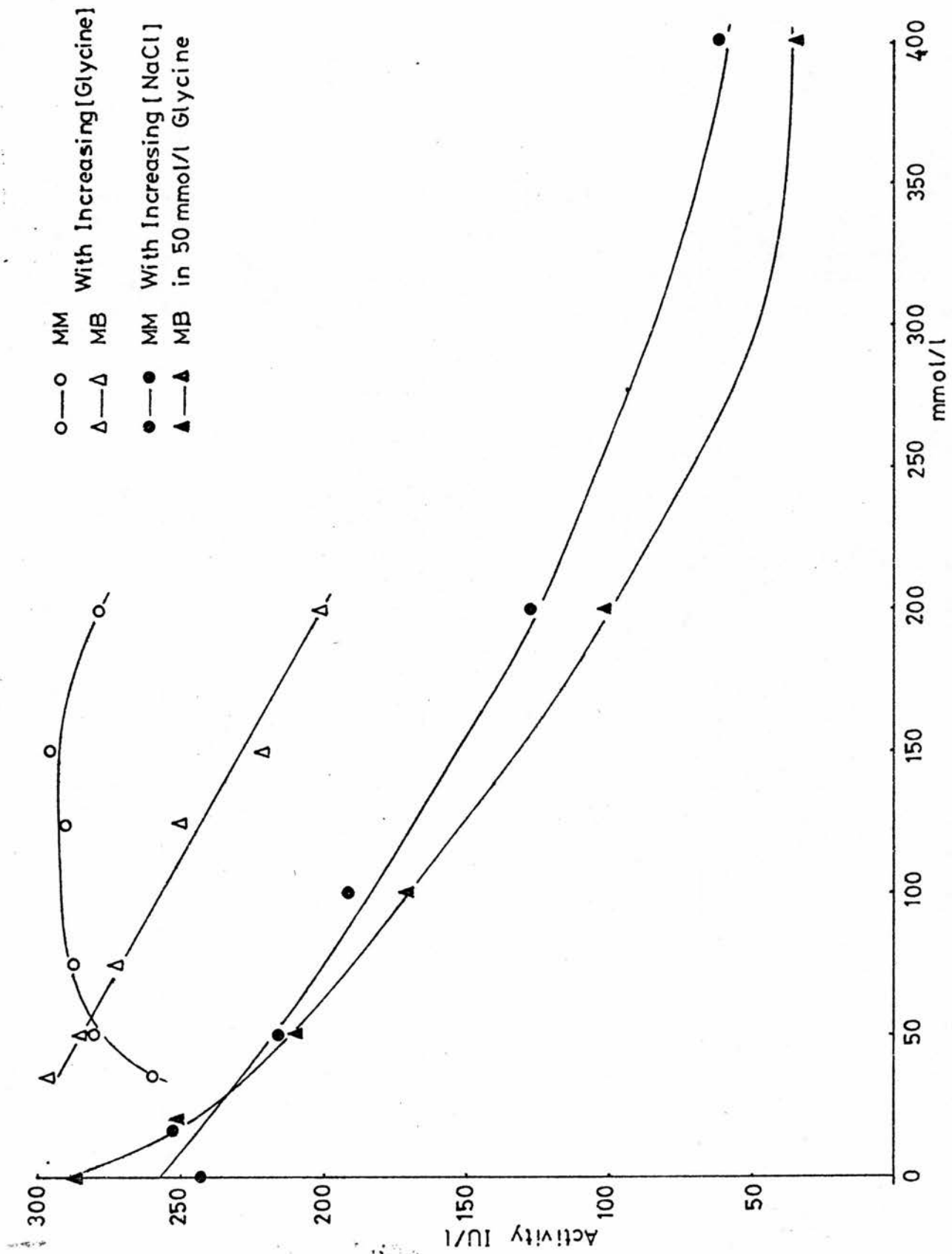
R = gas constant

T = temperature  $^{\circ}\text{K}$

C = a constant

Figure 5-18

Effect of [Buffer] and [NaCl] on the Activity of Creatine Kinase Isoenzymes



If  $\log k$  is plotted against  $1/T$ ,  $E_a$  can be obtained from the slope of the plot.  $E_a$  is related to the heat of activation ( $\Delta H_a$ ) by the equation,  $\Delta E_a = \Delta H_a + RT$ .

In an enzyme reaction; the maximum velocity ( $V$ ) can be used to replace  $k$  in the Arrhenius plot as  $V = kE_t$ ,  $E_t$ , the total enzyme concentration, being a constant value.

The Arrhenius plot is linear over only a limited range of temperature and only if a single observed reaction step is being changed by changing temperature. Temperature-dependent equilibria between different forms of the enzyme or substrate exist in many enzyme systems, and in these cases, non-linear Arrhenius plots will be obtained.

#### Procedure.

The temperature-dependence of the enzyme reaction rates was studied in the temperature range  $20^\circ - 40^\circ\text{C}$  in both the forward (Tanzer and Gilvarg, 1959) and backward (Rosalki, 1967) reactions. The pH of the buffer was adjusted at each temperature to pH 9.0 in the forward reaction and pH 7.0 in the backward reaction.

The substrate concentrations used were: creatine 20 mmol/l, ATP 1.0 mmol/l and  $\text{Mg}^{2+}$  ions 2 mmol/l in the forward reaction; and creatine phosphate 35 mmol/l, ADP 1.5 mmol/l and  $\text{Mg}^{2+}$  ions 10 mmol/l in the backward reaction.

#### Results.

The temperature-dependence of both forward and backward reactions of the two isoenzymes is shown in Fig. 5-19.

The corresponding Arrhenius plots are all non-linear

Figure 5-19

Effect of Temperature on the Activity of Creatine Kinase Isoenzymes

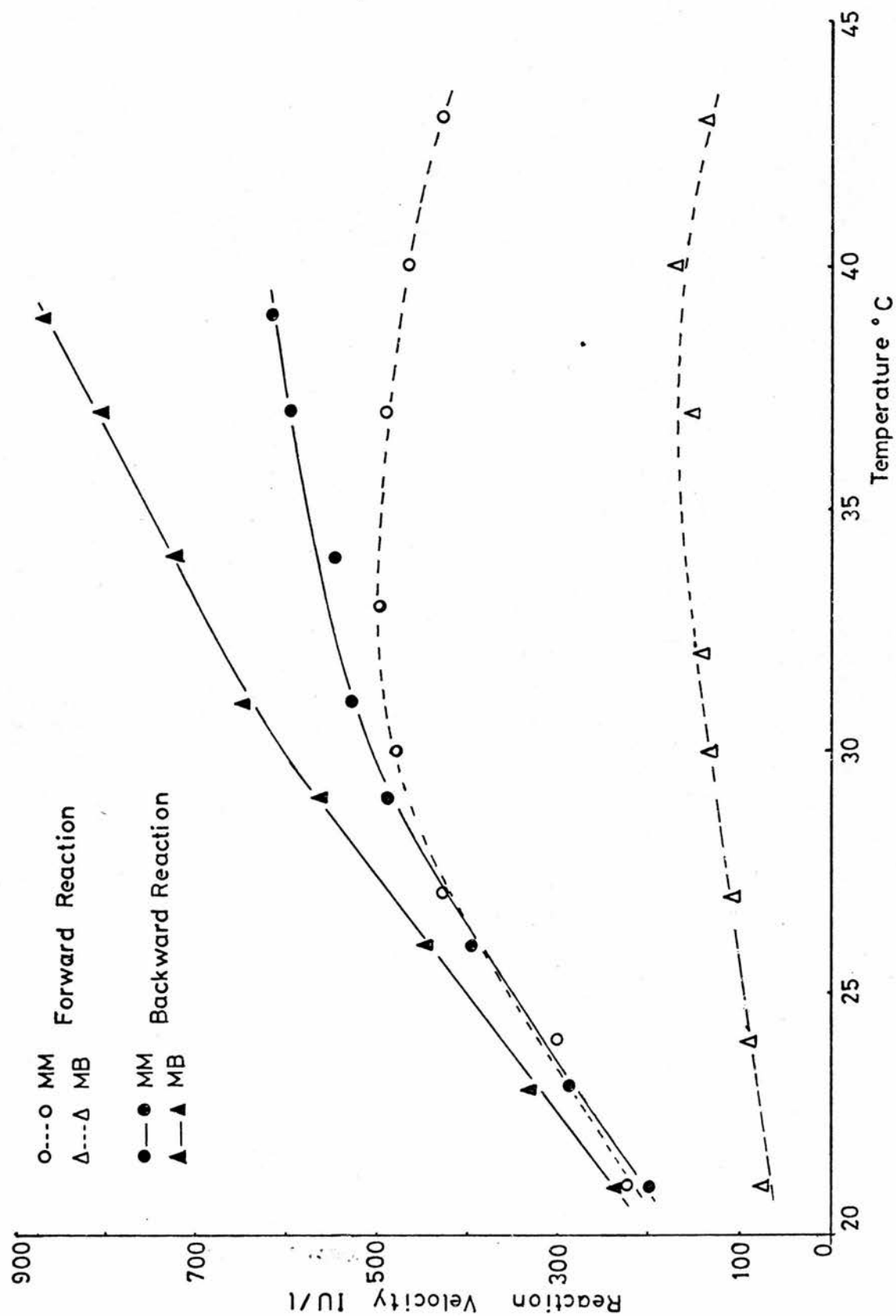
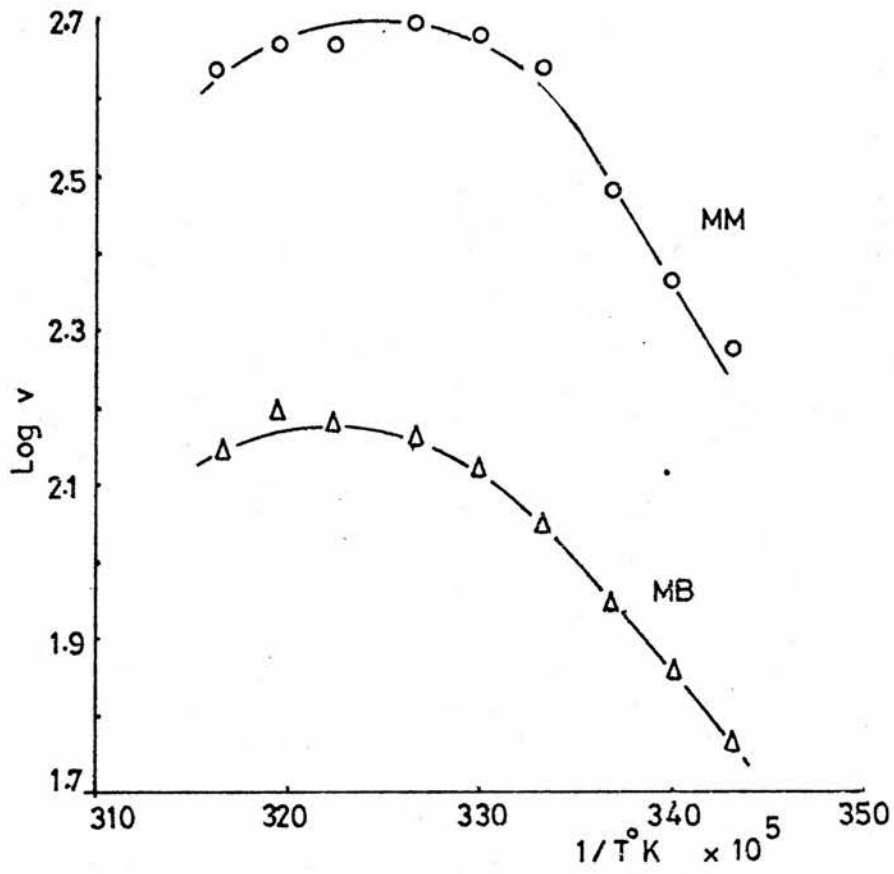


Figure 5-20

Arrhenius Plots for the MM and MB Isoenzymes

a. Forward Reaction



b. Backward Reaction

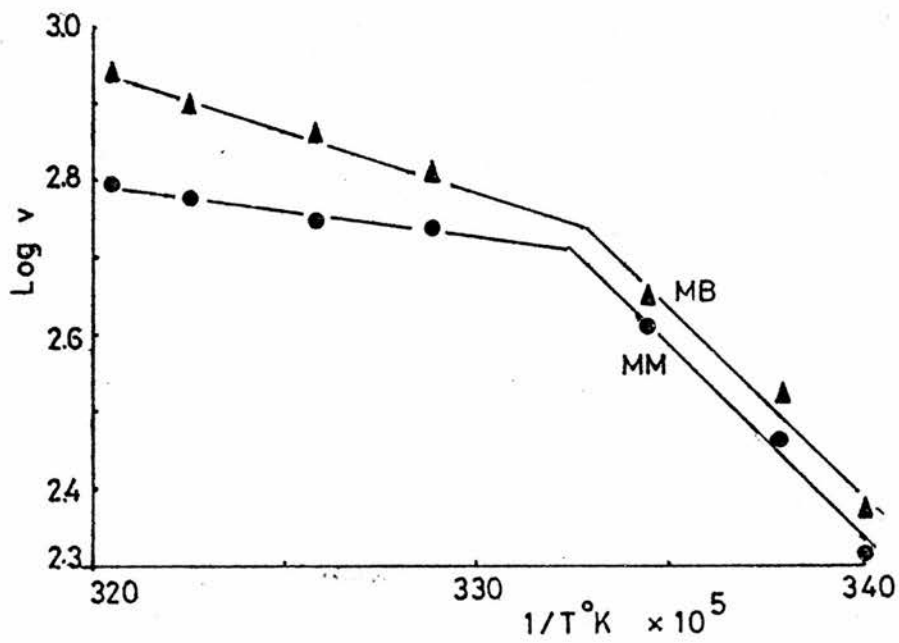


Table 5-7

The Effect of Temperature on the Reaction of Creatine Kinase  
Isoenzymes

	MB Isoenzyme	MM Isoenzyme
Forward Reaction		
$Q_{10}(25^{\circ} - 35^{\circ}\text{C})$	1.6	1.7
"E <sub>a</sub> "	3.2 Kcal (21 <sup>o</sup> - 30 <sup>o</sup> C)	9.3 Kcal (21 <sup>o</sup> - 30 <sup>o</sup> C)
Transition Temperature	30 <sup>o</sup> C	30 <sup>o</sup> C
Backward Reaction		
$Q_{10}(25^{\circ} - 35^{\circ}\text{C})$	1.9	1.6
"E <sub>a</sub> "	10.1 Kcal (20 <sup>o</sup> - 28 <sup>o</sup> C)	10.7 Kcal (20 <sup>o</sup> - 28 <sup>o</sup> C)
Transition Temperature	28 <sup>o</sup> C	28 <sup>o</sup> C

"E<sub>a</sub>" = apparent Arrhenius Energy

The forward reaction was measured by the method of Tanzer and Gilvarg (1959); the backward reaction by the Rosalki Method (1967).

with a change of slope at a transition temperature (Fig. 5-20a and b).

The apparent  $E_a$  and  $Q_{10}$  (i.e. change of reaction rate per  $10^\circ\text{C}$ ) values of the two isoenzymes are listed in Table 5-7.

#### Discussion.

In both forward and backward reactions, with the substrate concentrations used, the reaction velocity does not increase as much in the  $30^\circ - 40^\circ\text{C}$  range as in the  $20^\circ - 30^\circ\text{C}$  range, especially for the MM isoenzyme. The slight decrease in activity in the forward reaction of the MM isoenzyme beyond  $39^\circ\text{C}$  is likely to be due to substrate depletion as the enzyme is stable between  $39^\circ - 43^\circ\text{C}$ . Since the enzyme activities at all temperatures were measured at fixed substrate concentrations the temperature effects on the ionisation of substrates, and hence on the  $K_m$  values for the substrates, were also measured. Thus, the  $\log v$  against  $1/T$  plots, obtained in this study, give only apparent values of  $E_a$ .

5-3-6

#### Thiol activation.

It has been known for a long time that the inclusion of thiol compounds in the assay mixture for creatine kinase enhances the enzyme activity and increases accuracy and reproducibility (Hughes, 1962; Kar and Pearson, 1964). If the thiol compounds act by keeping the "essential"



thiol groups of the enzyme in reduced form, different degrees of activation by thiols with different redox potentials would be expected. Among the common low molecular weight thiols, the order of redox potentials at pH 7.0 is dithiothreitol < reduced glutathione < cysteine thioglycollic acid. Dithiothreitol, having the lowest redox potential, should be the most potent activator of the four. This agrees with a recent report of Rao et al. (1974), but is at variance with an earlier report (Kar and Pearson, 1964) which suggested that all the common low molecular weight thiols had a similar activation effect on creatine kinase.

The aim of the present experiment was to study the activation effect of five common thiol compounds (reduced glutathione, dithiothreitol, L-cysteine, thioglycollic acid and  $\beta$ -mercaptoethanol) on both the forward and backward reactions of the MM and MB isoenzymes.

#### Procedure and Results.

The enzyme activity was measured in the presence of 0-10 mmol/l thiol compounds by the method of Tanzer and Gilvarg (1959) at pH 9.0 at 37°C for the forward reaction and by the method of Rosalki (1967) at pH 7.0 at 37°C for the backward reaction. The activity was then plotted against the thiol concentration for both isoenzymes.

The pattern of activation by the thiol compounds for the MB isoenzyme was found to be similar to the pattern observed for the MM in both forward and backward reactions.

In the forward reaction, all thiol compounds, except cysteine, showed maximum activation from 2.5 mmol/l upwards. At low concentrations, cysteine inhibited both isoenzymes (Fig. 5-21a). In the backward reaction, the thiol compounds activated the isoenzymes to different extents: dithiothreitol >  $\beta$ -mercaptoethanol > reduced glutathione > L-cysteine > thioglycolic acid. Cysteine and thioglycolic acid at low concentrations inhibited the MM and to a smaller extent the MB isoenzymes (Fig. 5-21b).

Rao et al. (1975) reported that, in a mixture of the MM and MB isoenzymes, reduced glutathione only activated the MM isoenzyme while dithiothreitol activated both isoenzymes, this suggested that an assay solution using reduced glutathione as activator would underestimate serum creatine kinase activity. To determine whether or not reduced glutathione activates the MB isoenzyme in the presence of the MM isoenzyme, serum samples from patients with severe myocardial infarction and various mixtures of the purified MM and MB isoenzymes were assayed in the presence of i) 9 mmol/l reduced glutathione and ii) 9 mmol/l reduced glutathione + 10 mmol/l dithiothreitol. The backward reaction (Rosalki, 1967) was used for this study.

The results are shown in Table 5-8. In the present experimental conditions, dithiothreitol did not further activate the creatine kinase in serum samples which were shown by electrophoresis to contain the MB isoenzyme as well as the MM isoenzyme, nor did it further activate mixtures of the purified MM and MB isoenzymes. The

Figure 5-21

Activation of Creatine Kinase Isoenzymes by Thiol Compounds

a. Forward Reaction (MB Isoenzyme)

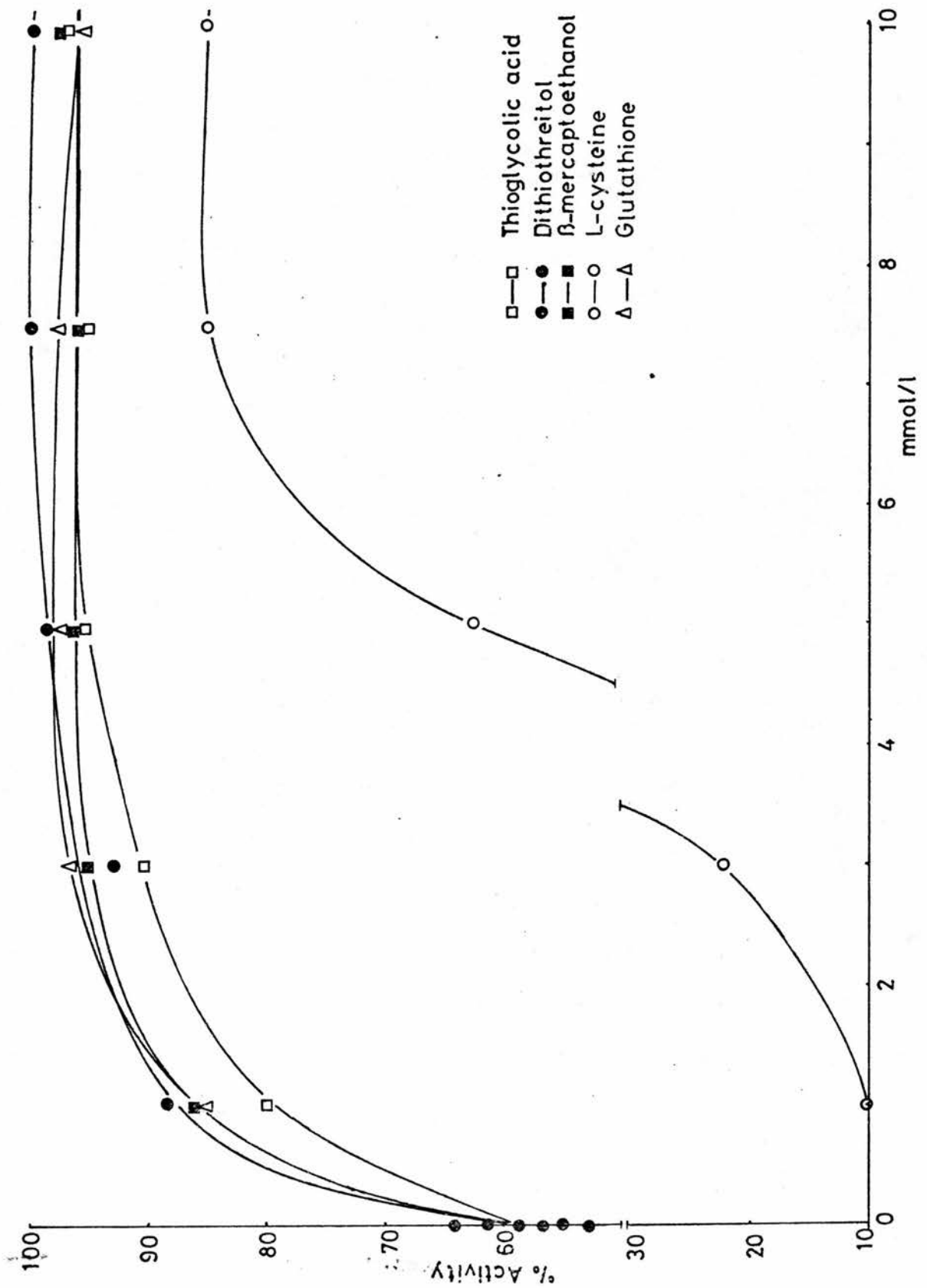


Figure 5-21

Activation of Creatine Kinase Isoenzymes by Thiol Compounds

b. Backward Reaction (MM Isoenzyme)

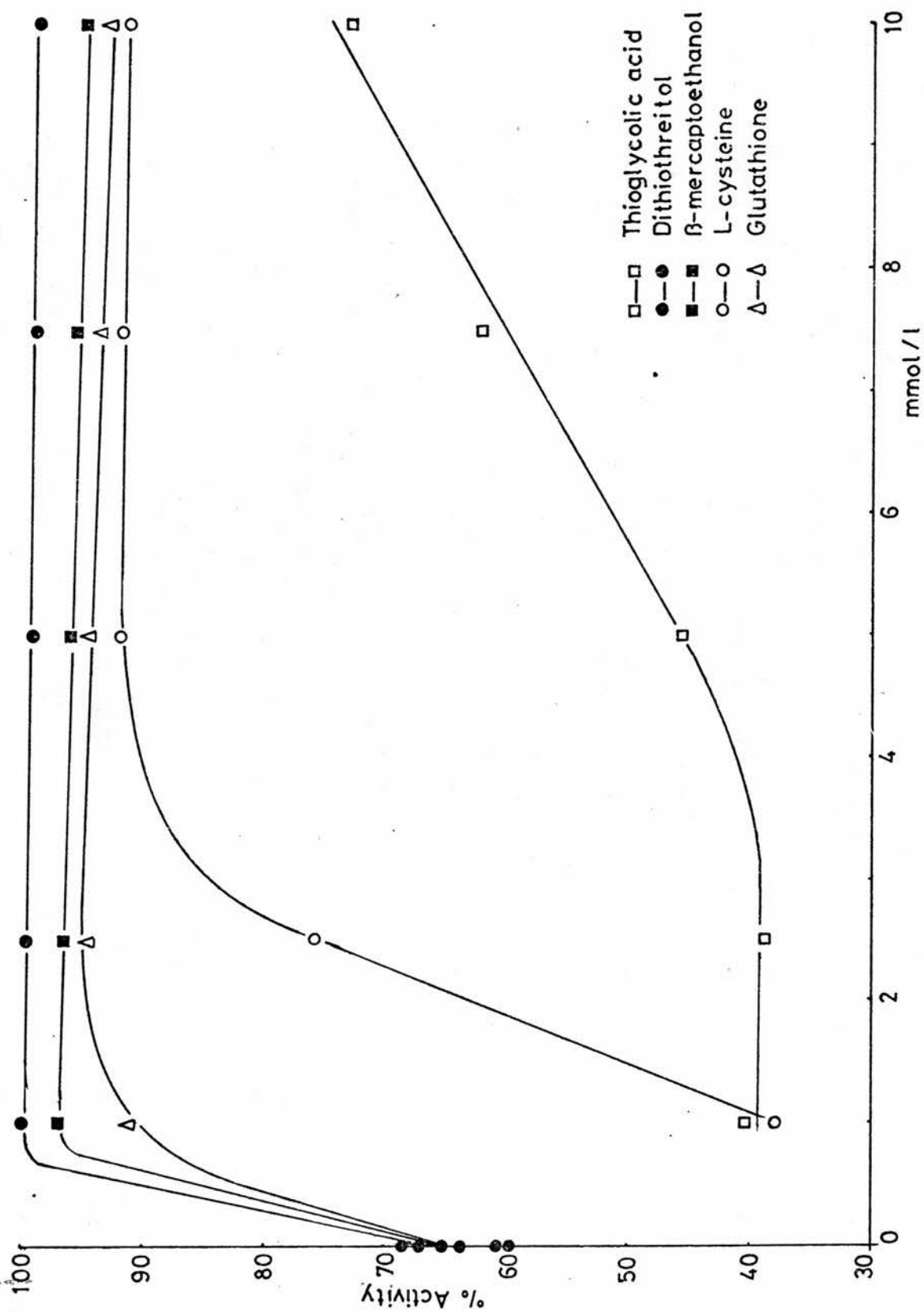


Table 5-8

Activation Effect on the Activity of the MM and MB Isoenzyme  
Mixture by Reduced Glutathione (GSH) and Dithiothreitol (DTT)

	Sample Activity (IU/l)									
<sup>+</sup> Sera with Myocardial Infarction	*264	(257)	2371	(2000)	1240	(1057)	1230	(1150)	1070	(921)
Purified MM Isoenzyme	285	(251)	596	(583)	1118	(1124)	-		-	
Purified MB Isoenzyme	224	(217)	515	(535)	1003	(1057)	-		-	
Mixture of Purified MM and MB Isoenzymes:										
Mixture Proportions	MM	MB	MM	MB	MM	MB	MM	MB	MM	MB
	976	+ 114	833	+ 224	596	+ 515	285	+ 779	142	+ 891
Observed Activity	1070	(1043)	1057	(1084)	1003	(989)	1030	(1084)	1016	(1000)

<sup>+</sup> All serum samples were shown by electrophoresis on agarose film to contain MB as well as MM isoenzyme activities.

\* Activity data without brackets were determined in the presence of 9 mmol/l GSH; those with brackets were determined in the presence of both 9 mmol/l GSH and 10 mmol/l DTT.

All enzyme activities were measured by the Rosalki method (1967).

presence of dithiothreitol neither enhanced nor inhibited the activity of the MM or MB isoenzymes when assayed separately with 9 mmol/l reduced glutathione.

### Discussion.

From the above experiments with individual isoenzymes the activation effect of the thiol compounds seems to be related to their respective redox potentials. This is more evident in the backward than in the forward reactions. The difference in activation between dithiothreitol,  $\beta$ -mercaptoethanol and reduced glutathione is, however, small and any one of the three thiols at a concentration in the range 3 to 10 mmol/l is suitable as activating reagent. Extrapolation of the present results with individual purified isoenzymes to conditions in serum may be misleading as serum contains many substances that react with thiols.

The assay conditions under which Rao et al. (1975) obtained differential activation of the MM and MB isoenzymes by glutathione and dithiothreitol must be critical, since the findings have not been reproduced in the present study using similar, but not identical, assay conditions.

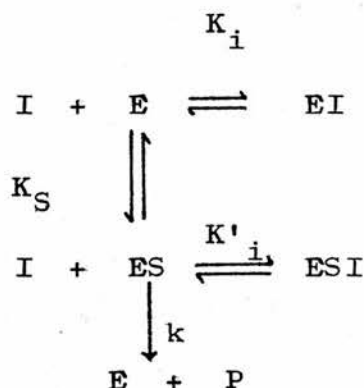
5-3-7

### Product inhibition.

Enzyme inhibition. Enzyme inhibition may be reversible or irreversible. The present study is concerned only with reversible inhibition where there is an equilibrium

between the inhibitor and the free enzyme or the enzyme-substrate complex.

In a simple one-substrate-one-intermediate system,



E = enzyme

S = substrate

I = inhibitor

P = product

$v_i$  = initial velocity

V = maximum velocity

$K_i$  = dissociation constant of EI

$K'_i$  = dissociation constant of ESI

$K_S$  = dissociation constant of ES

k = rate constant of rate determining step

$V_o$  = initial velocity in absence of inhibitor

The following rate equation can be derived:

$$\frac{1}{v_i} = \frac{V S}{K_S (1 + I/K_i) + (1 + I/K'_i) S}$$

$$\text{If } K_i = K'_i \quad \frac{1}{v_i} = \frac{V S}{(K_S + S)(1 + I/K_i)} \quad \dots \text{non-competitive inhibition}$$

If  $K'_i = \infty$   $\frac{1}{v_i} = \frac{V S}{K_S (1 + I/K_i) + S}$  .... competitive inhibition

If  $K_i = \infty$   $\frac{1}{v_i} = \frac{V S}{K_S + (1 + I/K'_i) S}$  .... uncompetitive inhibition

If  $K'_i > K_i$  or  $K_i > K'_i$  .... mixed inhibition

(Laidler and Bunting, 1973)

From the above equations it can be seen that 1. in non-competitive inhibition the presence of S has no effect on the binding of the enzyme with the inhibitor; 2. in competitive inhibition the presence of S reduces the enzyme binding with the inhibitor; and 3. in uncompetitive inhibition the inhibitor only binds with the enzyme-substrate complex.

Various graphical procedures have been proposed to discriminate between these types of inhibition. Some of these are

- i)  $1/v_i$  against  $1/S$
- ii)  $1/v_i$  against  $I$
- iii)  $S/v_i$  against  $S$
- iv)  $v_i$  against  $v_i/S$
- v)  $1/i$  against  $1/i$ ,  $i$  being  $(v_o - v_i)/v_o$
- vi)  $(I)(1-i)/i$  against  $S$

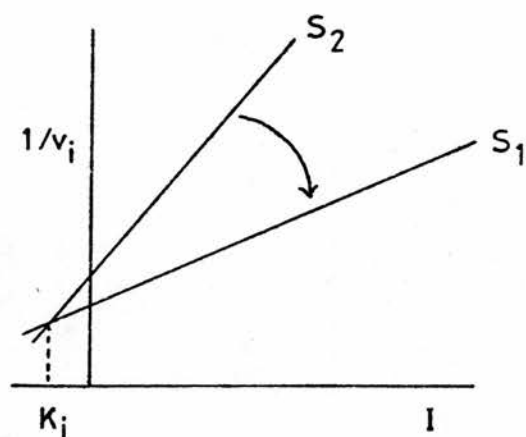
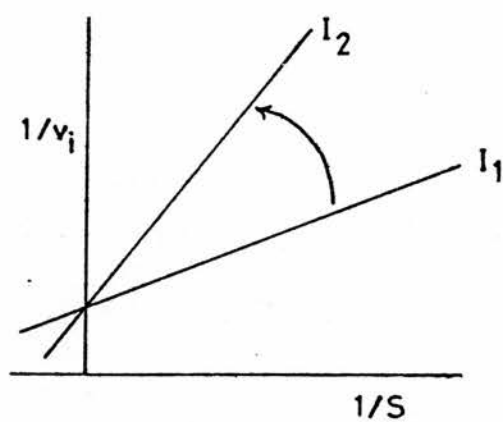
The graphical patterns of the various forms of inhibition of the first two kinds of plots are as follows:-



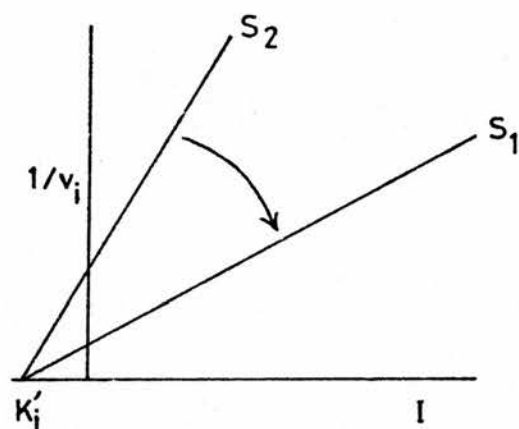
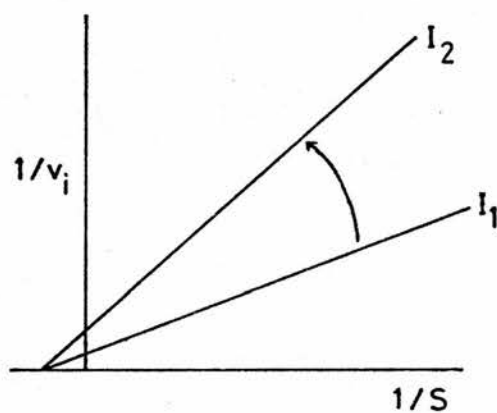
i)  $1/v_i$  against  $1/S$

ii)  $1/v_i$  against  $I$  (Dixon, 1953)

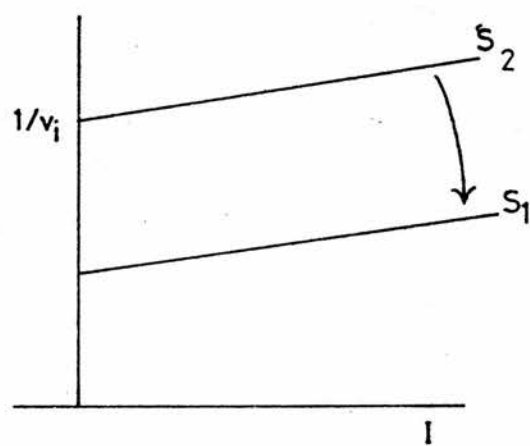
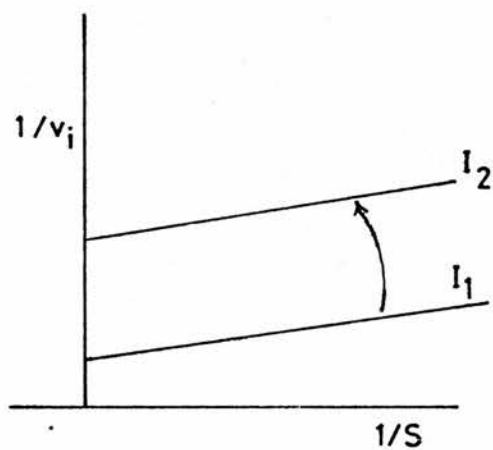
Pure competitive



Pure non-competitive



Pure uncompetitive



In the plot of  $1/v_i$  against  $I$  the inhibition constant ( $K_i$ ) can be read directly from the graph; whereas in the plot of  $1/v_i$  against  $1/S$ ,  $K_i$  can be obtained by further plotting the slopes against  $1/I$ .

In a two substrate system the  $K_i$  values may be found in a similar manner by holding one substrate concentration ( $S_a$ ) constant while varying that of the other ( $S_b$ ). The  $K_i$  for  $S_b$  obtained in this way might vary with the substrate concentration of  $S_a$ , depending on the reaction mechanism.

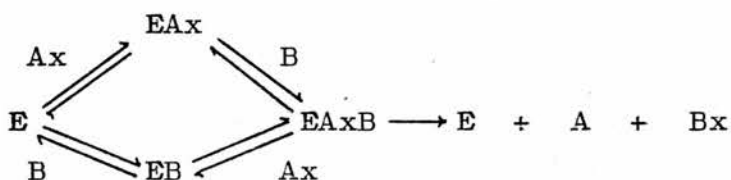
#### Inhibition by reaction products.

Reaction products may inhibit enzyme reactions by enhancing the backward reaction so as to compete with the forward reaction, or by combining with the enzyme to form inactive complexes. The former effect can usually be neglected as initial reaction velocities are usually measured. Also, in many enzyme reactions, the reaction equilibrium lies far to one side, the reverse reaction being insignificant. In these conditions, reaction products inhibit the enzyme reaction mainly by binding to the enzyme.

Since reaction products usually have structures similar to their substrate predecessors, they may compete with substrate for binding sites on the enzyme thus showing competitive inhibition.

The study of product inhibition patterns may help to clarify reaction mechanisms. In a rapid-equilibrium-

random-order type of mechanism for a reaction,  $Ax + B =$   
 $A + Bx$



$E$  = enzyme

$Ax, B$  = substrates

$A, Bx$  = products

Product A is a competitive inhibitor with respect to substrate Ax and product Bx to substrate B. . The inhibition pattern of product A in relation to substrate B (or Bx to Ax) is, however, complicated. It will be non-competitive if the binding of substrate B to the enzyme does not affect the subsequent binding to A; on the other hand, if the presence of B affects the binding of A to E, the inhibition will be of the mixed-type.

Since the substrate Ax binds to both E and EB, the product A being similar in structure to Ax can bind not only to E but also to EB as well. Therefore the  $K_i$  obtained for A is dependent on the concentration of B. Similarly  $K_i$  for Bx varies with the concentration of Ax. In this reaction mechanism there are, therefore, two possible enzyme-substrate-product complexes, E.A.B. and E.Ax.Bx which are incapable of reacting any further.

#### Procedure.

The product inhibition of creatine, creatine phosphate,  $MgATP^{2-}$  and  $MgADP^-$  for both isoenzymes was studied in

relation to the substrates. The conditions for the assay were:

Product inhibitor	Substrate concentration	Methods of Assay (37°C)
<b>1. Forward reaction</b>		
Creatine phosphate (0 - 15 mmol/l)	Creatine (2.5 - 15mmol/l) ATP (1.0 mmol/l) $Mg^{2+}/ATP = 2$	Tanzer and Gilvarg (1959) pH 9.0
ADP (0 - 1.5 mmol/l)	i) ATP (0.5 - 1.5 mmol/l) $Mg^{2+}/ATP = 2$ Creatine (20 mmol/l) ii) Creatine (7.5 - 25 mmol/l) ATP = 1.0 mmol/l $Mg^{2+}/ATP = 2.0$	Kuby (1954) pH 9.0
<b>2. Backward reaction</b>		
Creatine (0-15 mmol/l)	Creatine phosphate (0.67-5.0 mmol/l) ADP (1.0 mmol/l) $Mg^{2+}/ADP = 10$	Rosalki (1967) pH 7.0
ATP (0-2.0 mmol/l)	i) creatine phosphate (20 mmol/l) ADP (0.2-2 mmol/l) ii) ADP (1.0 mmol/l) creatine phosphate (0.5-20 mmol/l) Both i) and ii) $Mg^{2+}/ADP = 10$	Hughes (1962) pH 7.0

## Results.

The types of inhibition (as confirmed by both Lineweaver-Burk and Dixon plots) and the apparent  $K_i$  values (obtained from the Dixon plots) are listed in Table 5-9. Examples of the graphs obtained are shown in Fig. 22a,b,c and d.

## Discussion.

The patterns of product inhibition of both isoenzymes conform to the random-order-rapid-equilibrium type of mechanism. There were no qualitative differences between the isoenzymes although the " $K_i$ " values so obtained for MB isoenzyme were generally lower than the corresponding values of MM isoenzyme. The " $K_i$ " values are only apparent, being dependent on the concentration of the fixed substrates.

The inhibition of the forward reaction by ADP can be assumed to be due entirely to the species  $\text{MgADP}^-$  under the present assay conditions (see Section 5-3-1).

Little inhibition of the backward reaction by ATP (0-2 mmol/l) at pH 7.0 was observed although inhibition of the MM isoenzyme by  $\text{MgATP}^{2-}$  (0-3 mmol/l) has been reported at pH 8.0 (Morrison and James, 1965). In an early experiment, some inhibition of both isoenzymes by ATP was observed, giving non-linear Lineweaver-burk plots. Later it was found that the ATP solution used had hydrolysed, however, so the observed inhibition might have been due to the presence of inorganic phosphate present in the ATP solution. On repeating the experiments with a fresh

Table 5-9

Product Inhibition of Creatine Kinase Isoenzymes

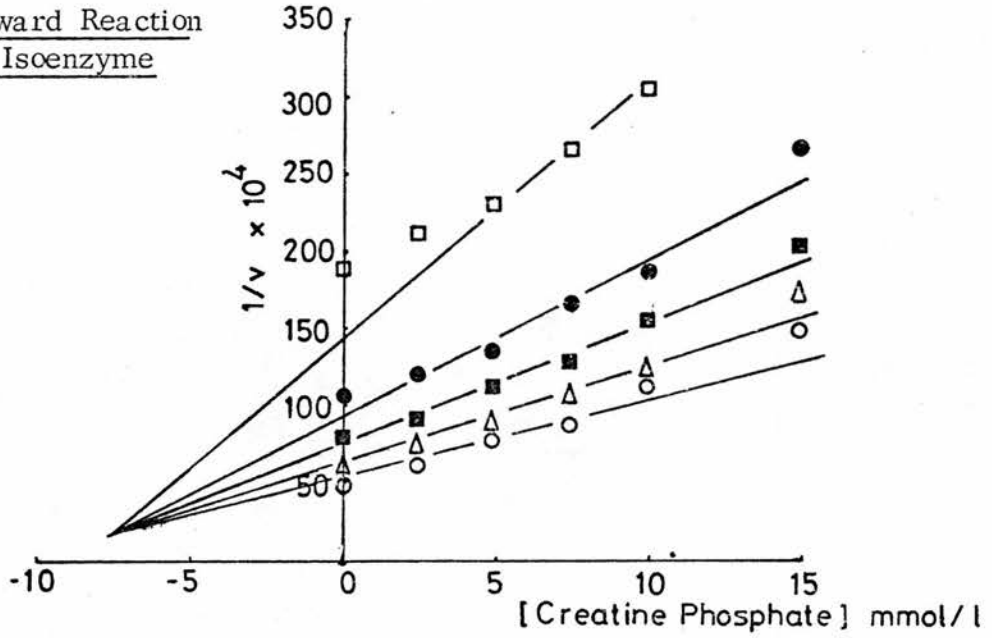
Product Inhibitor	Reference Substrate	pH	Type of Inhibition	
			MB Isoenzyme	MM Isoenzyme
MgADP	MgATP	9.0	Competitive ("K <sub>i</sub> " = 0.35 mmol/l)	Competitive ("K <sub>i</sub> " = 0.65 mmol/l)
	Creatine	9.0	Non-competitive ("K <sub>i</sub> " = 0.92 mmol/l)	Non-competitive ("K <sub>i</sub> " = 1.05 mmol/l)
Creatine phosphate	Creatine	9.0	Competitive ("K <sub>i</sub> " = 7.5 mmol/l)	Competitive ("K <sub>i</sub> " = 22 mmol/l)
MgATP	MgADP	7.0	Data Not Conclusive	
	Creatine phosphate	7.0	Data Not Conclusive	
Creatine	Creatine phosphate	7.0	Competitive ("K <sub>i</sub> " = 9.6 mmol/l)	Competitive ("K <sub>i</sub> " = 13.3 mmol/l)

"K<sub>i</sub>" = apparent inhibition constant.

Figure 5-22

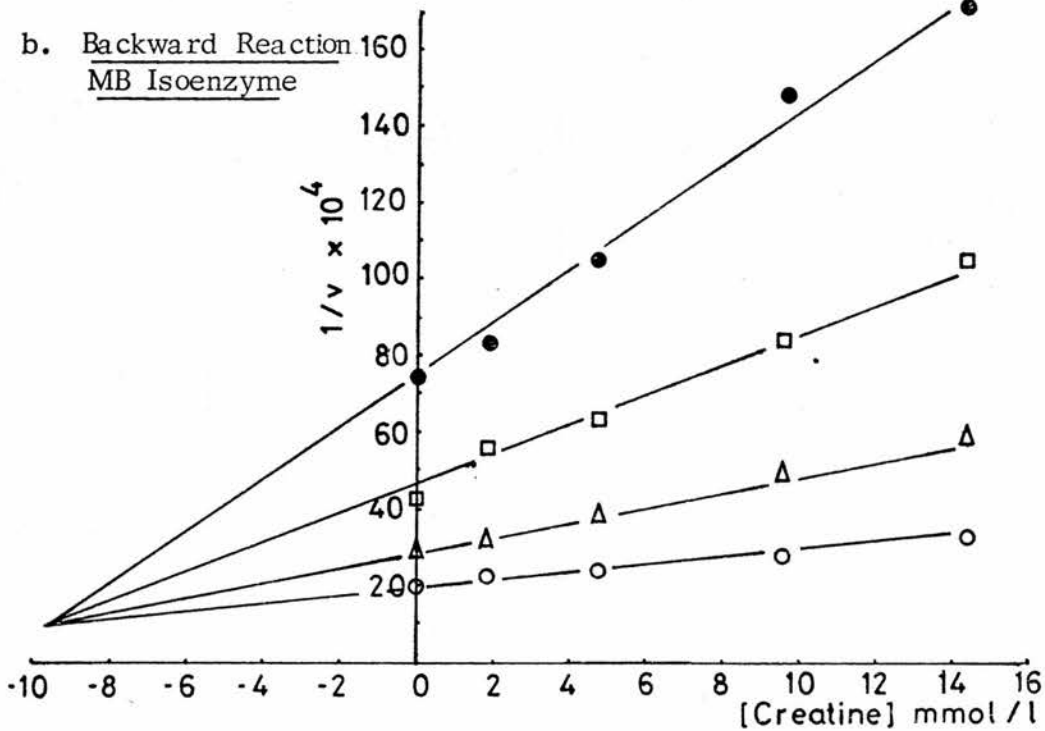
Product Inhibition of Creatine Kinase Isoenzymes

a. Forward Reaction  
MB Isoenzyme



[Creatine]:    □ 2.5 mmol/l;    ● 5.0 mmol/l;    ■ 7.5 mmol/l;  
                  Δ 10 mmol/l;    ○ 15 mmol/l.

b. Backward Reaction  
MB Isoenzyme



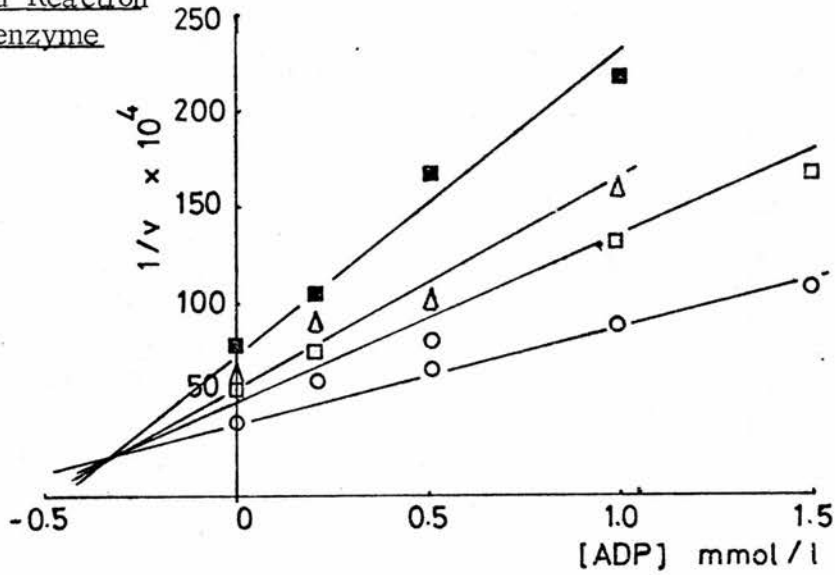
[Creatine phosphate]:

● 0.67 mmol/l;    □ 1.25 mmol/l;    Δ 2.5 mmol/l;    ○ 5.0 mmol/l.

Figure 5-22

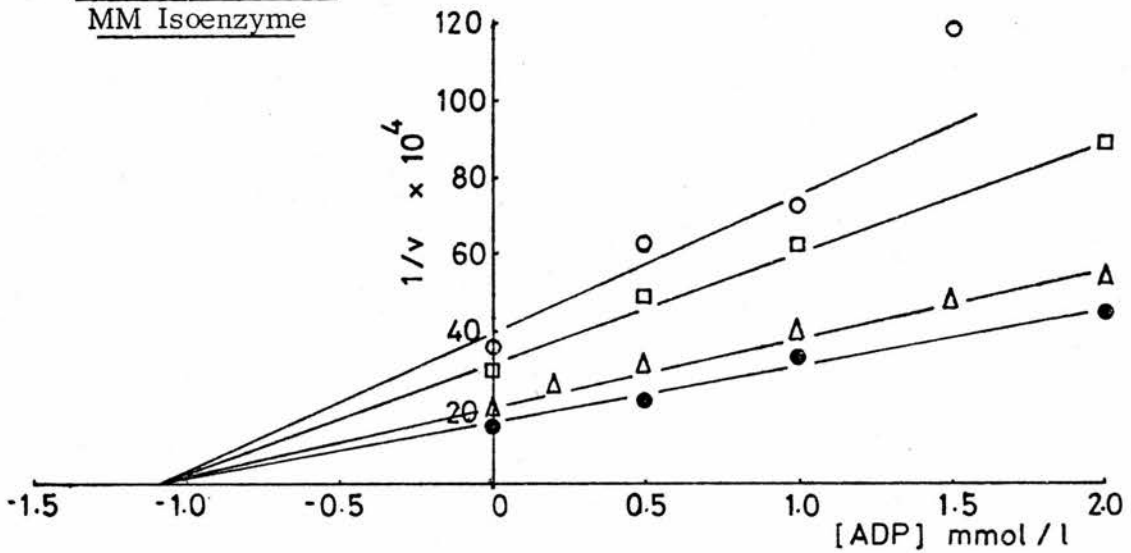
Product Inhibition of the Creatine Kinase Isoenzymes

c. Forward Reaction  
MB Isoenzyme



[ATP]: ■ 0.5 mmol/l; Δ 0.75 mmol/l; □ 1.0 mmol/l; ○ 1.5 mmol/l.

d. Forward Reaction  
MM Isoenzyme



[Creatine]:

○ 7.5 mmol/l; □ 10 mmol/l; Δ 20 mmol/l; ● 25 mmol/l.



sample of ATP, little inhibition of either isoenzyme was detected. Under the experimental conditions, nearly all the ATP existed as  $\text{MgATP}^{2-}$ , possibly with a very small amount of  $\text{MgHATP}^-$ . Since the affinity of the isoenzymes for  $\text{MgATP}^{2-}$  is much lower than that for  $\text{MgADP}^-$  (one tenth as low for the MB isoenzyme and one fifth as low for the MM isoenzyme; Table 5-6), it would be expected that a relatively high concentration of  $\text{MgATP}^{2-}$  was necessary to compete with  $\text{MgADP}^-$ . However, a concentration range 0-2 mmol/l  $\text{MgATP}^{2-}$  was considered sufficient, as other studies had shown considerable inhibition in this range (Morrison and James, 1965). The cause for this apparent discrepancy is not clear.

#### 5-3-8

#### Effect of anions.

Anions affect enzyme activities either by non-specific action by increasing the ionic strength or by specific individual effects. The specific effects of anions include i) changing the ionisation constants of enzymes or substrates, ii) altering enzyme structure, iii) binding with enzyme or enzyme-substrate complex as ligands, iv) affecting the charge-distribution of the active sites of enzymes, or v) functioning as competitive inhibitors to substrates. Thus they can, directly or indirectly, alter the kinetics of enzyme reactions. The effects of ten different anions on the activities of the two isoenzymes has been investigated in the present study.

## Procedure and Results.

1. 50 mmol/l solutions of nitrate, chloride, flouride, borate, acetate, benzoate, sulphate, phosphate, succinate and maleate as sodium salts were incubated separately with the isoenzymes in the assay solutions. The pH of the salt solution was preadjusted to that of the assay mixture, pH 9.0 for the forward reaction measured by the method of Tanzer and Gilvarg (1959) and pH 7.0 for the backward reaction (Rosalki, 1967). Two concentrations of enzymes were used in each case to eliminate the possibility of the observed effect being due to the coupling enzymes involved in the assay. The inhibition was expressed as a percentage of the activity in the absence of the anion.

There were few differences between the isoenzymes for all the anions studied. With the exception of borate, which only inhibited the forward reaction, all other anions affected both the forward and backward reactions to a similar extent. Three types of anionic effect were found i) strong inhibition (< 70% residual activity) by benzoate, sulphate, phosphate, maleate and borate (forward reaction), ii) mild inhibition (70%-95% residual activity) by chloride, nitrate, fluoride and succinate, iii) no inhibition by acetate (Table 5-10).

2. The effect of phosphate ions was chosen to study in more detail, as the enzyme reaction is concerned with phosphate transfer, and inorganic phosphate is most likely to be present as an impurity in the adenine nucleotide

Table 5-10

Anionic Inhibition on the Activity of Creatine Kinase Isoenzymes

Anions 50 mmol/l	% Residual Activity			
	Forward Reaction		Backward Reaction	
	MB Isoenzyme	MM Isoenzyme	MB Isoenzyme	MM Isoenzyme
Water	100	100	100	100
Nitrate	69	78	86	84
Chloride	83	83	91	99
Fluoride	86	94	86	85
Borate	28	34	91	101
*Acetate	99	112	95	103
Benzoate	44	38	65	66
Sulphate	43	53	54	69
Phosphate	37	50	42	54
Succinate	80	100	88	96
Maleate	58	70	60	75

\* The total concentration of acetate was 53 mmol/l because of the presence of magnesium acetate in the assay solution.

substrates. The inhibition by phosphate ions was studied in the concentration range 0-62 mmol/l in the presence of varying concentrations of both substrates, creatine phosphate and  $\text{MgADP}^-$ , in the backward reaction. The results were analysed by the Lineweaver-Burk and Dixon plots.

The type of inhibition by inorganic phosphate (as shown by both Lineweaver-Burk and Dixon plots) with respect to the substrates are listed in Table 5-11. Some of the results are shown in Fig. 5-23a and b.

### Discussion.

Comparison of the effects of different anions at a fixed concentration can only give an approximate estimate of the relative inhibitory action of the anion, as the degree of inhibition may not be linearly proportional to the concentration of the anion.

The finding that  $\text{Cl}^-$ ,  $\text{NO}_3^-$  and  $\text{F}^-$  are mild inhibitors is slightly misleading, for they are only so in the absence of either creatine or  $\text{MgADP}$ . In the presence of both creatine and  $\text{MgADP}$ , these anions can greatly stabilise the complex creatine-enzyme- $\text{MgADP}$  by occupying the gap between creatine and  $\text{MgADP}$  in the complex (Milner-White and Watts, 1971).

The acetate ion hardly inhibited either the forward or the backward reactions, although  $K_i$  values varying from 350-400 mmol/l have been reported (Heyde and Morrison, 1970; Nihei et al., 1961). Acetate has also been reported

Table 5-11

Inorganic Phosphate Inhibition on the Backward Reaction of  
Creatine Kinase Isoenzymes

Reference	Substrate	Types of Inhibition	
		MM Isoenzyme	MB Isoenzyme
	Creatine Phosphate	Non-competitive	Non-competitive
		("K <sub>i</sub> " = 30 mmol/l)	("K <sub>i</sub> " = 48 mmol/l)
	MgADP	Non-competitive	Competitive (?)
		("K <sub>i</sub> " = 45 mmol/l)	("K <sub>i</sub> " = 20 mmol/l)

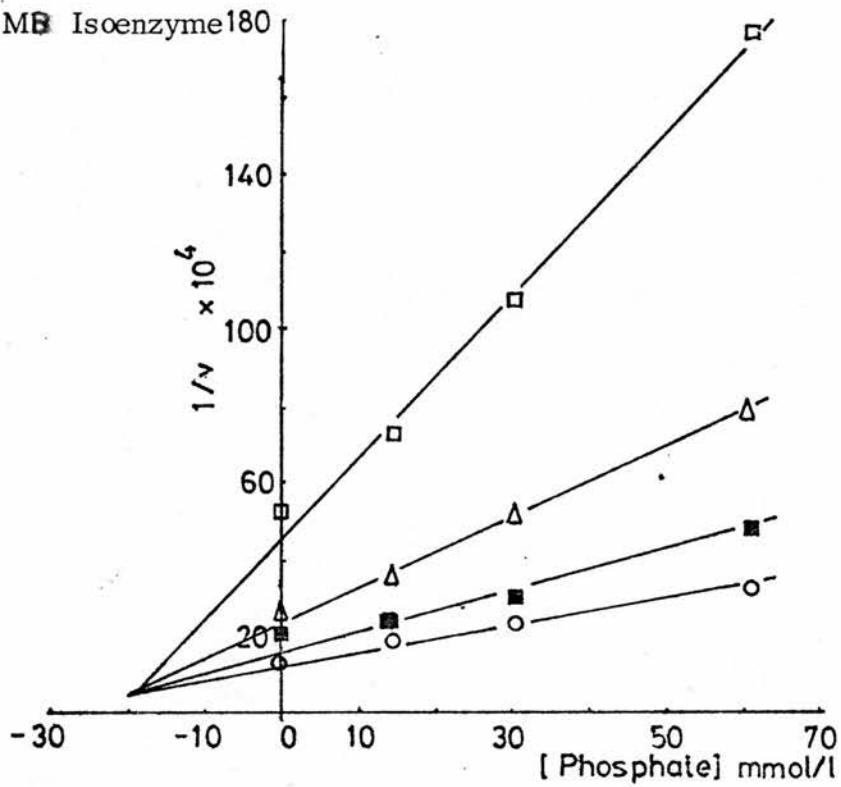
"K<sub>i</sub>" = apparent inhibition constant.

Figure 5-23

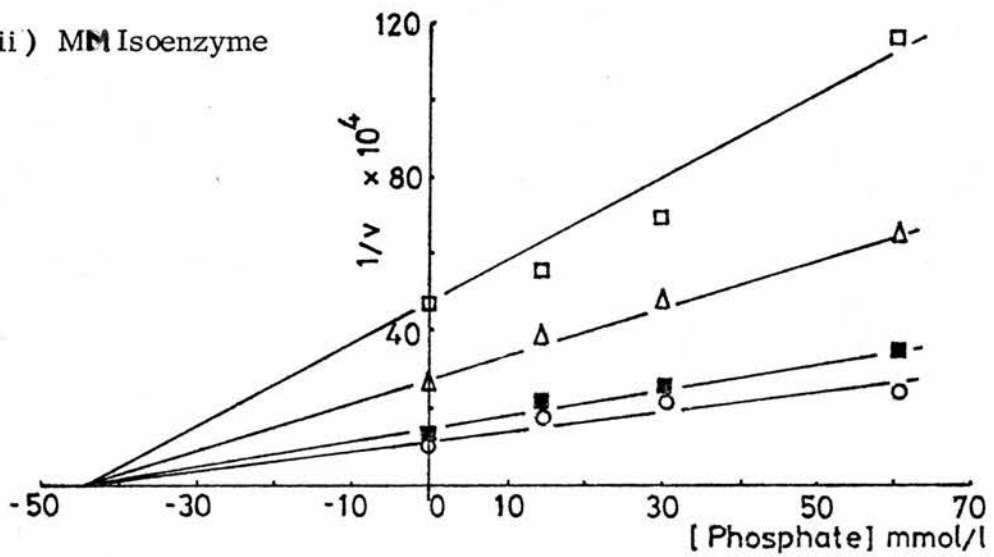
Inorganic Phosphate Inhibition of the Activity of Creatine Kinase  
Isoenzymes in the Backward Reaction

a. With Respect to Substrate ADP

i) MB Isoenzyme



ii) MM Isoenzyme



[ADP]:

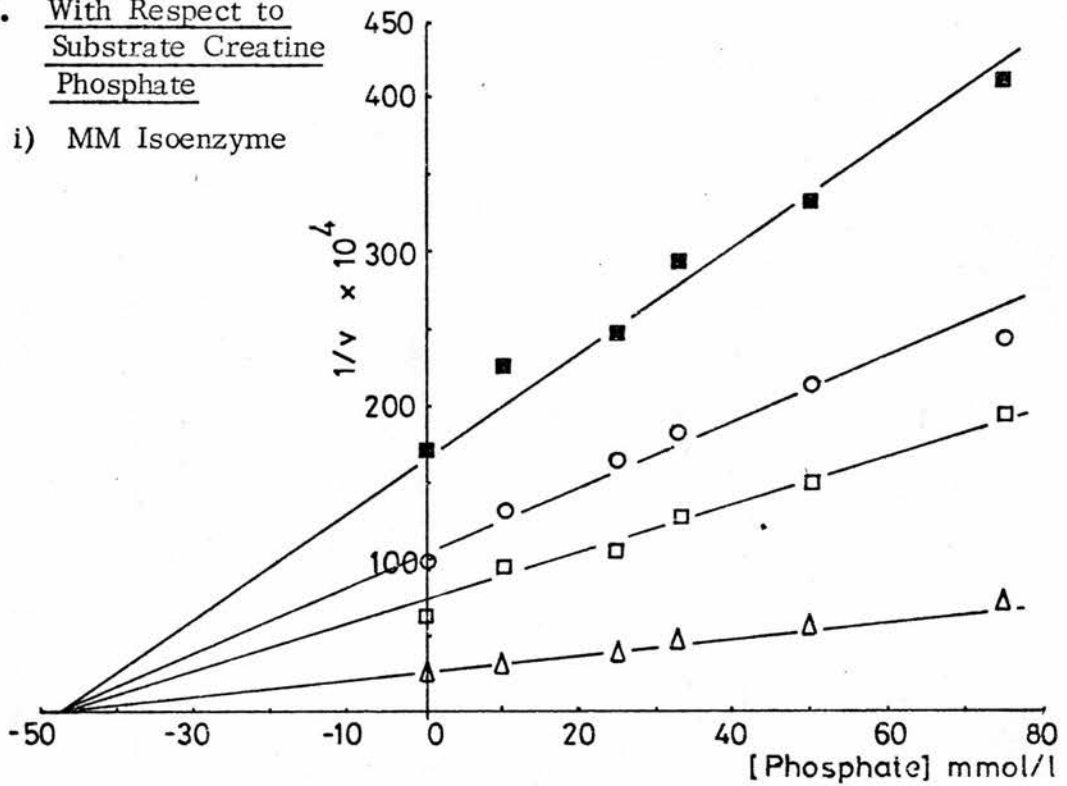
- 0.25 mmol/l;    Δ 0.5 mmol/l;    ■ 1.0 mmol/l;  
○ 2.0 mmol/l.

Figure 5-23

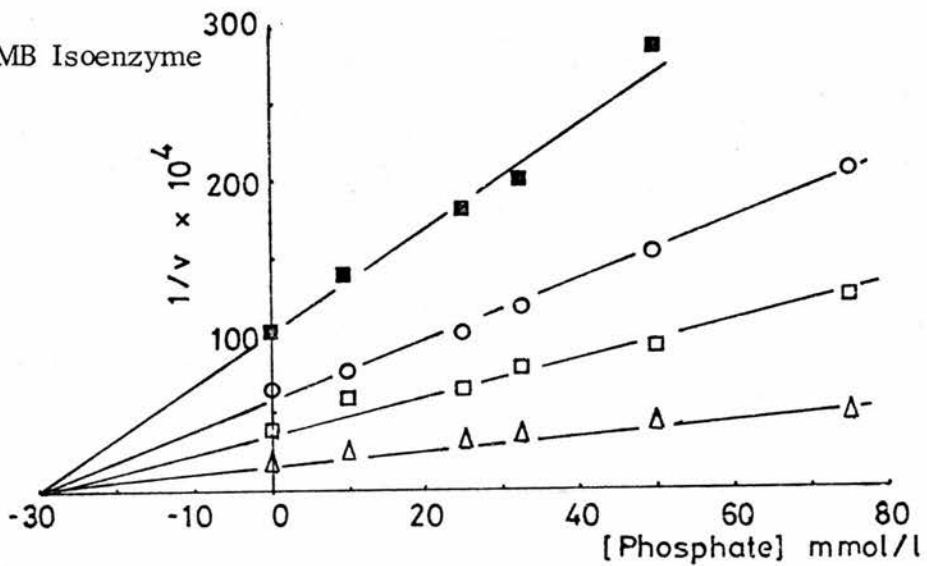
Inorganic Phosphate Inhibition of the Activity of Creatine Kinase Isoenzyme in the Backward Reaction

b. With Respect to  
Substrate Creatine  
Phosphate

i) MM Isoenzyme



ii) MB Isoenzyme



[Creatine phosphate]:

■ 0.625 mmol/l; ○ 12.5 mmol/l; □ 2.5 mmol/l; Δ 10 mmol/l.

to activate creatine kinase by 10-30% (Milner-White and Watts, 1971). It has been proposed that acetate binds to the enzyme at an allosteric site (Watts, 1973).

Nihei et al. (1961) suggested that the strong inhibition by sulphate and phosphate was due to their binding with the enzyme at the site occupied by the transferable organic phosphoryl group. Thus, these anions would be expected to act as competitive inhibitors in relation to the phosphorylated substrate (Creatine phosphate in the backward reaction). For phosphate ions, however, a non-competitive (or mixed) inhibition was found with both MB and MM isoenzymes. This may be attributable to the conformational changes occurring after the binding of the phosphate ion (Watts, 1973).

5-4

#### Discussion.

The present survey has shown that the two isoenzymes are qualitatively very similar. Most of the substrate-affinity and product inhibition studies suggest that the MB isoenzyme has the same reaction mechanism as the MM isoenzyme. Both isoenzymes had similar pH optima, and both were similarly inhibited by different types of anions, reaction products, iodoacetamide, oxidised glutathione and excess  $Mg^{2+}$ . Under suitable conditions both could be reactivated on dilution after treatment with urea, guanidine/HCl and p-hydroxymercuribenzoate and both were activated by thiols. These similarities might have been



forecast, for half of the MB molecule is of the muscle type.

However, despite the similarities there were many quantitative differences between the isoenzymes. Apart from the difference in electrophoretic mobility the MB isoenzyme had a higher affinity for both MgADP and creatine phosphate. Furthermore, the isoenzymes differ in the way which the  $K_m$  value for MgADP and for creatine phosphate varied with pH. The activity of the MB isoenzyme was enhanced by glycine/NaOH buffer relative to tris/HCl whereas the MM isoenzyme was not. Thirdly, in many circumstances, including i) stability at extreme pH values ii) heat inactivation and iii) urea inactivation, the MB isoenzyme has been shown to be less stable than the MM isoenzyme. The only occasion in this study where MB isoenzyme was apparently more stable than MM isoenzyme was during the pHMB inactivation.

Because of the relatively unstable character of the MB isoenzyme and its similarity to the MM isoenzyme in many other properties, the differential measurement of the MB isoenzyme in the presence of the MM isoenzyme, as in clinical diagnosis, is difficult. Using the differences in kinetic parameters noted above it is possible by using low and high substrate concentrations at suitable pH values to enhance the activity of the MB and MM isoenzymes relative to one another. By determining the ratio of creatine kinase activity at low to high substrate concentrations, the proportion of MB isoenzyme present in a mixture

of MM and MB isoenzymes may be calculated (Witteveen et al., 1974). However, there are difficulties in measuring creatine kinase activity at low substrate concentrations and it is difficult to measure creatine kinase activity sufficiently precisely to detect the relatively low proportions of the MB isoenzyme present in most normal and pathological sera.

In those instances where quantitation of isoenzyme mixtures has been possible, the most successful techniques have employed differential inhibition or denaturation of the different isoenzymes, e.g. the effect of heat and urea denaturation on lactate dehydrogenase. For creatine kinase, however, these are less likely to be successful with the MB isoenzyme since this is usually present in much smaller quantity than the MM isoenzyme in serum. Anionic inhibitors had similar effects on both isoenzymes and the differences in product inhibition were not great enough to form the basis of any practical method. Heat inactivation at 50°C affected the MB much more than the MM isoenzyme and in view of the small quantity of the MB isoenzyme present in serum, it is doubtful whether its selective destruction by heat could be made a practical method of measuring the MB isoenzyme.

Inactivation by pHMB, which affected the MM more than the MB isoenzyme, might be of value in the selective measurement of the MB isoenzyme. However, the rate of inactivation by pHMB was found to be critically dependent on the type and concentration of buffer. Moreover, the

inactivated enzymes were readily reactivated on dilution in the assay mixture which contained a thiol compound. Therefore, although it is possible to devise conditions capable of measuring the MB isoenzyme in mixtures of partially purified MB and MM isoenzymes, these conditions might not apply to the analysis of mixtures of creatine kinase isoenzymes in serum.

No differences between the isoenzymes in their activation by different thiol compounds have been found, but this aspect requires further study as serum creatine kinase isoenzymes might behave differently from their purified forms from tissue. A method has been recently reported (Rao et al, 1975) describing the selective assay of the MB isoenzyme based on the differential activation of two thiol compounds.

On the basis of the present findings, it seems unlikely that any of the differences observed between the isoenzymes, in kinetic behaviour, inactivation and inhibition by various substances, is great enough to warrant attempts at its development as a routine test for determining the MB isoenzyme in serum. Currently, it seems that techniques based on differences in charge, such as electrophoresis or ion-exchange chromatography, are more likely to provide simple and reliable techniques for the accurate determination of MB isoenzyme activity in serum. This will be further discussed in the following section.

SECTION 6

DETERMINATION OF THE MB ISOENZYME OF  
CREATINE KINASE IN SERUM

This section is concerned with the search for a convenient and simple method of measuring the MB isoenzyme in serum.

6-1

### Introduction.

The measurement of clinically useful isoenzymes has been of importance in clinical chemistry for a long time. The methods used can, in general, be classified into two groups:

1. Methods involving the removal of interfering isoenzymes by either i) separation techniques or ii) differential inactivation.

2. Methods using the original untreated isoenzyme mixture in conditions that suppress the activities of the interfering isoenzyme(s) without affecting the activity of the isoenzyme to be measured. Methods in categories 1 ii) and 2 may be difficult to develop and depend on the physical and enzymatic properties of the isoenzymes concerned. Methods in category 1 i) are more common and many isoenzymes were discovered by methods of this type, particularly electrophoresis.

The following is a brief summary of the efforts made, mostly in the past 10-20 years, to develop methods for the differential measurement of isoenzymes, especially those of lactate dehydrogenase. It seemed possible that similar methods might be used to assay the MB isoenzyme.

Method 1: Removal of interfering isoenzymes.

i) Separation techniques. Ion-exchange chromatography and electrophoresis, techniques based on differences in charge, are the most commonly employed methods in this category. The physical forces involved are summarised in Table 6-1. Electrophoresis is often the technique first used to demonstrate the heterogeneity of an enzyme.

Wieme, in 1959, used agar gel electrophoresis to assay the lactate dehydrogenase isoenzymes. Electrophoretic methods using other media, such as cellulose acetate and starch, were soon used for serum isoenzyme analysis. Hess and Walter (1961) were among the earliest to introduce an ion-exchange method using DEAE cellulose to separate lactate dehydrogenase isoenzymes.

ii) Destruction of interfering isoenzymes.

a) Selective precipitation of isoenzymes. This is based on the different solubilities of the isoenzymes and has been used as a routine procedure e.g. the precipitation of the slower forms of lactate dehydrogenase by acetone (Latner and Turner, 1963) and of liver alkaline phosphatase by ethanol (Peacock et al., 1963).

b) Heat inactivation. Herbert (1944) found that prostatic acid phosphatase was inactivated after one hour at 37°C while erythrocyte acid phosphatase was little affected by this treatment. She was probably the first to use heat stability as a means of differentiating enzyme species. Since then, heat treatment has been used to measure LD<sub>1</sub> and LD<sub>2</sub> (LD<sub>1-5</sub> = isoenzymes of lactate dehydrogenase in descending order of mobility towards the anode on electrophoresis)

Table 6-1

Physical Forces Involved in the Chromatography and Electrophoresis  
Separation Techniques

Separation Method	Impelling Forces $F_1$	Retarding Forces $F_2$	Predominant Force for Separation	Separation Depending on
Ion-exchange Chromatography	Hydrodynamic	Electrostatic Polarizability and at times Molecular Sieving Effects	$F_2$	Ionic Nature; Molecular Dimension
Electrophoresis in Porous Supporting Medium	Electrostatic	Molecular Friction, Electrokinetic Surface Energy	$F_1$	Ionic Nature

(after Morris and Morris, 1963)

which are more heat resistant than LD<sub>4</sub> and LD<sub>5</sub>. Alkaline phosphatases from bone and liver have also been differentiated in this way (Whitby and Moss, 1975).

c) Immunological methods. Because isoenzymes have different protein structures, antisera can, in principle, be raised specifically against each isoenzyme. By selectively inactivating a particular species, the activity of the other species can therefore be measured e.g. the MM and BB isoenzymes of creatine kinase (Jockers-Wretou and Pfeleiderer, 1975).

Method 2: Suppression of interfering isoenzyme activities.

i) Differences in substrate affinity. This method is well exemplified by the lactate dehydrogenase isoenzymes. The fast-moving forms (LD<sub>1</sub> and LD<sub>2</sub>) have a higher substrate affinity for pyruvate than the slower forms (LD<sub>4</sub> and LD<sub>5</sub>). Plagemann et al. (1960) calculated the ratio of the velocity at high substrate concentration to the velocity at low substrate concentration, and plotted the logarithm of this ratio against the percentages of LD<sub>1</sub> and LD<sub>5</sub> in a mixture of the two isoenzymes. A direct relationship was found and there was excellent agreement between observed and calculated values. Differences in pH optima between isoenzymes have also been used, in combination with the difference in substrate affinity, e.g. in the measurement of the relative percentages of the isoenzymes of aspartate aminotransferase (Fleisher et al., 1960).

ii) Use of substrate analogues. An isoenzyme may have a characteristic ratio of activity with substrate to



its activity with a substrate analogue. For example, lactate dehydrogenase isoenzymes have characteristic ratios of activity with pyruvate to their activity with 2-oxobutyrate (Wilkinson and Rosalki, 1960). These ratios have been used to indicate the relative proportions of the different isoenzymes present.

iii) Use of inhibitors. Abul-Fadl and King (1949) showed that L(+) tartrate inhibited the acid phosphatase of the prostate gland, while formaldehyde and copper inhibited other acid phosphatases. Urea and oxalate, which inhibit the slow and fast moving forms of lactate dehydrogenase respectively, have also found a number of applications in selective measurements of lactate dehydrogenase isoenzymes. Difficulties in measuring the MB isoenzyme.

The chief difficulty is that only small amounts of the MB isoenzyme are present even in the serum of patients with acute myocardial infarction - usually less than 30% of the total creatine kinase activity. In normal serum the difficulties are much greater, since less than 5% of the total serum creatine kinase is present as the MB isoenzyme. Moreover, the MB isoenzyme has been shown, under a variety of conditions, to be less stable than the more abundant MM form (see Section 5). Thus, many conventional means of inhibition or inactivation often affect the MB more than the MM isoenzyme. Methods based on the difference between total activity and that of MM isoenzyme after the destruction of the MB isoenzyme are not practicable because of the small amount of the MB isoenzyme present and the serum dilution effect (see Section 2).

Existing methods of measuring the MB isoenzyme.

In view of the difficulties mentioned above it is hardly surprising that most of the existing methods are based on the separation by charge differences between the MM and MB isoenzymes.

Electrophoresis in a variety of media has been widely used in many laboratories - cellulose acetate (Roberts et al., 1974), agarose (Sherwin, 1967), agar (Van der Veen and Willebrands, 1966), starch gel (Sjovall and Voigt, 1964) and polyacrylamide gel (Smith, 1972), followed by various methods of demonstrating and quantitating the separated isoenzymes. Simple methods using column chromatography on DEAE Sephadex (Mercer, 1974) and DEAE cellulose (Nealon and Henderson, 1975) have been described.

A method using the difference in substrate affinity between the MM and MB isoenzymes has been reported (Witteveen et al., 1973). An immunological method designed mainly to differentiate the MM and BB isoenzymes has also been used to measure the MB isoenzyme (Jockers-Wretou, 1975). More recently, it has been reported (Rao et al., 1975) that differential activation by thiol compounds may be used to measure the amount of the MM and MB isoenzymes in serum. In 1974 when this work was started the MB isoenzyme was most commonly measured by electrophoretic separation. None of the electrophoretic methods was altogether satisfactory. They were either too cumbersome or too imprecise for the routine quantitative analysis of the isoenzyme. It was hoped that a simple batch ion-exchange method might

help solve this problem.

## 6-2

### Methods.

All creatine kinase activities were determined by the method of Rosalki (1967) using reagents supplied by the Boehringer Corporation, Lewes, England. Enzyme activity was determined at 37°C at 340 nm using an LKB reaction rate analyser; the total assay volume was 1.1 ml and the sample volume (for serum) was 25 µl (see Section 2).

Partially purified preparations of the MM and MB isoenzymes from heart tissue (see Section 5) were used for assessment of the methods.

### 6-2-1

#### Ion-exchange chromatography.

Ion-exchange chromatography separates the components in a mixture on the basis of the number of charged groups available on each molecule for interaction with the ion-exchanger. Since the two isoenzymes of creatine kinase differ widely in electric charge, the simple procedure of stepwise elution may be used instead of the more elaborate gradient elution. For simplicity a batch method was chosen in preference to a column method. In this study Sephadex (cross-linking dextran) was used rather than cellulose, mainly because of the higher exchange capacity of Sephadex, due to the presence of more ionisable groups within the matrix of the gel beads. Another advantage

that Sephadex gives is that its bead form enables it to be sedimented readily, which makes it convenient for a batch technique involving washing and packing. The main disadvantage in using Sephadex is the large changes in volume which result from changes in salt concentrations or pH. It is possible that large molecules, which have gained access to the gel matrix during the initial stages of elution when the salt concentration is low, might be trapped when the gel shrinks as the salt concentration rises during the process of elution.

Since the MB isoenzyme is more negatively charged than the MM isoenzyme, a cationic exchanger which binds the MM isoenzyme in preference to the MB isoenzyme should, in principle, be used so as to yield the MB isoenzyme on a single elution with buffer of appropriate pH and ionic strength. This was tried with carboxymethyl Sephadex (CM-50, Pharmacia, Uppsala, Sweden). No satisfactory separation was achieved, due to difficulty in finding a suitable pH value for the eluting buffer. The isoelectric point of the MM isoenzyme is around pH 5.6, but lowering of the buffer pH to below 6.0 is not feasible because both isoenzymes are unstable at pH values below 6.0 (Section 5) and the CM Sephadex is not fully effective below pH 6.0. Therefore, a less direct two-step elution technique using the anion exchanger, DEAE Sephadex, was used.

#### Reagents.

1. Buffer 1: Tris/HCl, 50 mmol/l, pH 8.0 in NaCl  
0.09 mol/l

2. Buffer 2: Tris/HCl, 50 mmol/l, pH 8.0 in NaCl 0.25 mol/l
3. DEAE Sephadex A-50 (Pharmacia Fine Chemicals, Uppsala, Sweden)

About 10g DEAE Sephadex was equilibrated with Buffer 1, with three changes of buffer within 24 hours. Excess buffer was poured off and the slurry distributed in 2.5 ml portions, equivalent to about 100 mg dry Sephadex, into 10 ml plastic tubes which were stored at 4°C.

#### Procedure.

1 ml of serum or diluted serum (active samples were diluted to give a creatine kinase activity of 600 IU/l) was added with 5 ml Buffer 1 to a 2.5 ml portion of DEAE Sephadex. Preliminary dialysis of serum samples was not found to be necessary as the ionic strength of serum is roughly equal to that of Buffer 1. The tube was mixed by inversion and allowed to stand for 15 min. After spinning, the supernatant was decanted and the sediment washed twice (four times in certain experiments) with 6 ml portions of Buffer 1. The washings were pooled to give eluate 1 which contained the MM isoenzyme (see results).

The Sephadex sediment was then washed three times with Buffer 2, using 4 ml, 3 ml and 3 ml volumes and pooling the washings to give eluate 2 (containing the MB isoenzyme). The creatine kinase activity of eluate 2 was determined spectrophotometrically using 250  $\mu$ l sample in place of the usual 25  $\mu$ l.

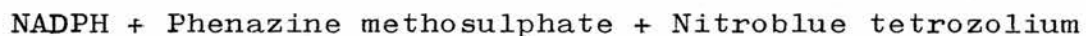
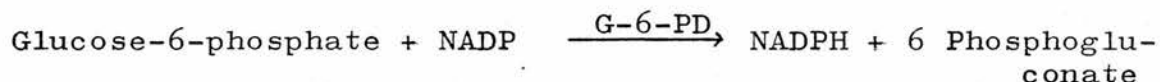
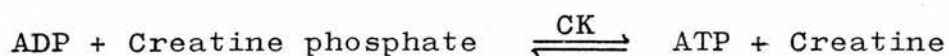
6-2-2 Separation on polyacrylamide gel slabs (Smith, 1972).

Polyacrylamide gel offers a stable and satisfactory medium for protein separations. The gel is formed by the polymerisation of acrylamide monomer and bisacrylamide in the presence of free radicals. The polymerisation is normally catalysed by either the photodecomposition of riboflavin or ammonium persulphate in the presence of tetramethylethylenediamine (TEMED) in the absence of excess oxygen.

The polyacrylamide gel consists of long chain molecules with cross linkages, the pore size in the gel being dependent on the concentration of the acrylamide and bisacrylamide. With a range of total acrylamide concentration between 6.5 and 20%, the average pore diameter varies from 0.6 to 4 nm (Fawcett and Morris, 1966). This enables the gel to exert a sieving effect on most proteins whose diameters vary from 1.6 to 8 nm, and thereby give sharp and well resolved bands. The polyacrylamide gel does not contain any ionisable groups and hence endosmosis is negligible. It is transparent and so provides a good medium for staining. However, polyacrylamide absorbs light in the UV region, so the detection of UV absorbing substances in the gel is often not practicable. The tight matrix of the gel allows little diffusion of protein macromolecules into and out of the gel. Thus, for demonstration of enzyme activity in the gel by coupled enzyme reactions, the coupling enzymes have to be introduced into the gel by electrophoresis.

Creatine kinase (CK) activity can be demonstrated in

the gel by the following coupled enzyme reactions:



G-6-PD = Glucose-6-phosphate dehydrogenase

HK = hexokinase

The formazan is insoluble in water but soluble in organic solvents, stable to oxygen, but can be decomposed in strong light. It has a higher extinction coefficient at 560 nm than NADPH has at 340 nm and so increases the sensitivity of the method. The intensity of the formazan band is determined by a number of factors - the enzyme concentration, the substrate concentration, the incubation temperature, the incubation period and the diffusion coefficients of the reacting components.

The method to be described is that originally reported by Smith (1972). Some modifications were made to optimise the method for quantitative measurements. This method was used throughout the clinical study on the release of creatine kinase isoenzymes and details of its performance have already been described in Section 4.

#### 6-2-3 Separation on agarose film.

Agarose film (1% agarose) is a medium with a large



pore size ( 120nm in diameter), offering little steric hindrance. Therefore, it requires only a short time and a low voltage for the electrophoretic separation of isoenzymes. The procedure can be carried out at room temperature with little danger of overheating. Since the gel is porous it allows entry of coupling enzymes into the gel by diffusion. Also, since the agarose gel has no charged groups, endosmosis is minimal. For these reasons a much simpler procedure is possible with this medium than with polyacrylamide gel slabs. However because of the softness and thinness of the gel, the film is much more delicate to handle than the tough polyacrylamide gel slab.

Unlike polyacrylamide gel, agarose gel does not absorb UV light. Therefore demonstration of creatine kinase activity by NADPH fluorescence is possible. Fluorescence measurements are often much more sensitive than equivalent spectrophotometric measurements and the intensity of emitted light is directly proportional to the total number of fluorescent molecules present, provided the number of molecules is small, and so, unlike transmission measurement of the stained bands, a homogeneous distribution of fluorescent molecules in the band is not essential for a valid reading of fluorescence intensity. Despite these advantages, fluorescence measurements are easily affected by the impurities in the gel, the physical state of the fluorescing NADPH, the intensity of the exciting beam, the thickness of the gel and the ambient temperature.



### Equipment.

A commercially available electrophoresis system was used (Corning Eel, Evans Electroselenium Ltd., Halstead, Essex, England).

1. A cassette electrophoretic cell and power supply ( $90V \pm 5\%$ ).

The cell consisted of a base and cover. The cell base was divided into two chambers each with a carbon electrode. The cell cover had a U-shaped cassette holder to secure the agarose film during electrophoresis.

2. Plastic-backed pre-prepared agarose-film.

This consisted of a thin layer of agarose gel ( $481 \mu m$ ) adherent to a transparent plastic backing. Sample wells capable of holding  $2.3 \mu l$  solution were imprinted in the gel surface.

3. An incubator-drier.

An incubator section was maintained at  $39^{\circ}C \pm 2^{\circ}C$ ; the drier section at  $60^{\circ}C \pm 5^{\circ}C$  with a draught of filtered air.

### Reagents.

1. Sodium barbital buffer 50 mmol/l, pH 8.6.
2. Agarose gel consisting of 1% W/V agarose and 5% W/V sucrose in 30 mmol/l 2-amino-2-methyl-1-propanol buffer, pH 8.6.
3. Creatine substrate mixture
  - a) 50 mmol/l 2-(N-morpholino) ethane sulphonic acid  
pH 6.2.
  - b) bottles of lyophilised reagent-mixtures: ADP,  $MgCl_2$ ,

L-cysteine, AMP, glucose, hexokinase, NAD, G-6-PD  
and creatine phosphate

4. Substrate solution, formed by dissolving one bottle of Reagent 3b) with 1 ml of Reagent 3a).

Procedure.

The procedure advised by the manufacturers was followed.

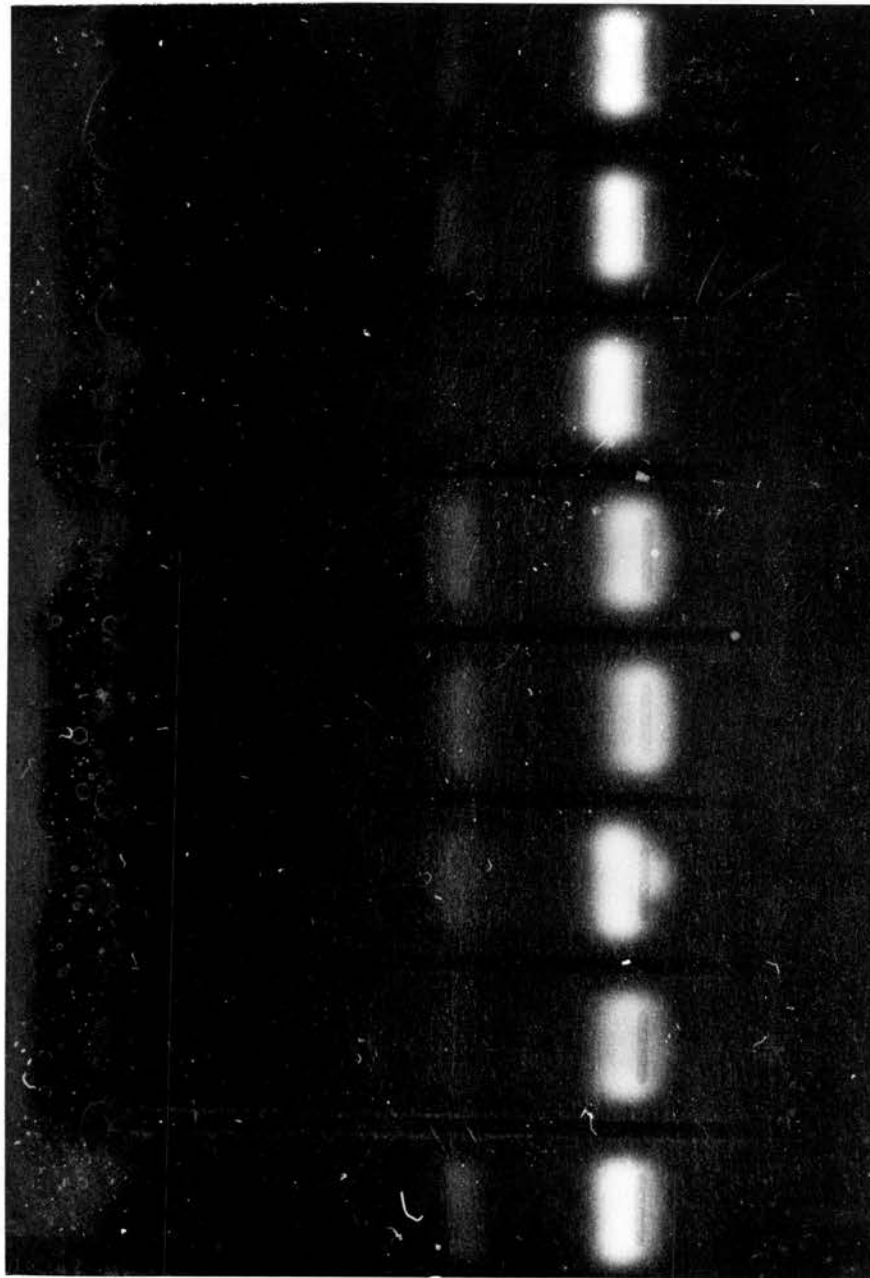
2  $\mu$ l sample was introduced into each sample well and allowed to absorb into the gel before the film was mounted onto the cell cover. After a 20 min electrophoretic run, the gel was removed from the cell and a layer of substrate solution was spread evenly on the gel surface with the help of a 5 ml serological pipette. The same coupled reaction sequence was used to demonstrate the isoenzymes as in the polyacrylamide method except that NADPH fluorescence was used for visualisation. The gel was incubated at 39°C for 20 min and then dried at 60°C for 20 min.

When viewed under UV light, fluorescent bands indicated the position of creatine kinase isoenzymes (Fig. 6-1). These bands were scanned for NADPH fluorescence with the Vitatron TLD 100 densitometer using a mercury source with an ultraviolet filter in the primary position and a 458 nm filter in the secondary position. The MB isoenzyme content of the sample was calculated as in the polyacrylamide method above.

The optimal activity of serum samples for this method was found to be 300 IU/l and any samples with activity higher than 300 IU/l were diluted accordingly.

Figure 6-1

Demonstration of Serum Creatine Kinase Isoenzymes by Electrophoresis  
on Agarose Film



+

↑

MB

Isoenzyme

↑

MM

Isoenzyme

-

6-2-4 An attempt to assay the isoenzyme by differential inactivation.

In Section 5, the MB isoenzyme was shown to be more stable than the MM isoenzyme to inactivation by p-hydroxy-mercuribenzoate (pHMB). It was, therefore, considered that this reagent might be of value in the analysis of the MB isoenzyme. If this inactivation effect was coupled with the differences between the isoenzymes in substrate affinity, the activity of MM isoenzyme might be almost completely suppressed, thereby allowing the MB isoenzyme be determined independently.

Various amounts of the MM and MB isoenzyme preparations were separately incubated for 60 min at 4°C in a mixture containing 14 mmol/l pHMB and 30 mmol/l tris/HCl, pH 7.0. The activity of the isoenzymes was measured immediately after the incubation, the reaction being started by introducing 25 µl of the incubation mixture. The creatine phosphate concentration in the substrate solution was reduced from the usual 35 mmol/l to 10 mmol/l. The latter concentration was optimal for the MB isoenzyme, but suboptimal for the MM isoenzyme. The results are shown in Table 6-2. It can be seen that the activity of the MM isoenzyme was suppressed to 2% while 48% of the MB isoenzyme still remained.

Normal serum samples were fortified with various amounts of both isoenzyme preparations and were incubated and assayed in exactly the same way as above, the serum samples being diluted five times on mixing with the incubation mixture. Table 6-3 shows that between 37% and 45% of the

Table 6-2

Inactivation of the Partially Purified Creatine Kinase Isoenzymes  
by 14 mmol/l pHMB at 4°C for 60 min

	MM Isoenzyme				MB Isoenzyme			
Original Activity IU/l	2500	1500	500	560	490	542	550	540
Activity after Incubation	50	20	10	7	261	254	250	254
% Residual Activity	2%	1.4%	2%	1.5%	53.3%	47%	46%	47%

Table 6-3

Inactivation of the Creatine Kinase Isoenzymes in Serum by  
14 mmol/l pHMB at 4°C for 60 min

Serum Sample	Total MM Activity IU/l	Total MB Activity IU/l	Activity after Incubation IU/l	% Residual Activity	
				MM Isoenzyme	*MB Isoenzyme
1	3743	0	1332	35.6	-
2	2455	0	1016	41.4	-
3	1255	0	559	44.5	-
4	531	0	231	43.5	-
5	98	736	446	-	55.3
6	52	490	282	-	53.2
7	25	245	147	-	55.9
8	30	98	99	-	68.4

\* calculated on the assumption that the MM component activity had decreased to 40%.

MM isoenzyme activity remained; there was a recovery of 53% to 68% of MB isoenzyme activity. Thus, the differential inactivation of the MM isoenzyme seen with purified isoenzyme preparations was not observed in serum.

6-3                      Assessment of methods.

6-3-1                    Batch ion-exchange method.

Accuracy.

Varying amounts of the partially purified MM and MB isoenzymes were added to normal sera whose MB isoenzyme activity was assumed to be negligible. About 95% (Table 6-4) of added MM isoenzyme was present in eluate 1, and eluate 2 contained no creatine kinase activity providing the original specimen (after dilution) showed less than 600 IU/1 (two washes to obtain eluate 1) or 1500 IU/1 (four washes to obtain eluate 1).

Addition of MB isoenzyme to normal sera with low creatine kinase activity (Table 6-5) showed that an average of 75% of the applied MB activity was recovered in eluate 2. Increase of NaCl concentration to 0.3 mol/l in Buffer 2, increase in the number of washings and the presence of 2 mmol/l  $\beta$ -mercaptoethanol in the eluting buffers did not alter the recovery. In some of these experiments, eluate 1 contained more creatine kinase activity than was present in the original sample. Presumably this was partly due

Table 6-4

DEAE Sephadex Ion-exchange Method - Effect of Addition of Varying  
Amounts of MM Isoenzyme to Serum Samples of Low Total CK Activity

Serum Sample	Total MM Activity IU/l	Total MB Activity IU/l	Total Activity of 1st Eluate IU/l	Total Activity of 2nd Eluate IU/l	MM Re- covery in 1st Eluate
*1	2500	0	2200	20	89%
2	2500	0	2300	14	91%
3	2500	0	2500	0	100%
4	1750	0	1740	4	99%
5	1740	0	1620	5	93%
6	1830	0	1780	0	97%
7	1240	0	1300	0	105%
8	1260	0	1170	0	92%
9	1290	0	1060	0	82%
*10	890	0	830	12	93%
11	720	0	690	6	96%
12	480	0	460	0	96%
13	390	0	420	0	108%
14	250	0	240	0	96%
15	220	0	200	0	91%

\* For samples 1 - 9: eluate 1 obtained from 4 washes and for samples 10-15 : eluate 1 obtained from 2 washes.

Table 6-5

DEAE Sephadex Ion-exchange Method - Effect of Addition of MB  
Isoenzyme to Serum Samples of Low Total CK Activity (Assumed  
to be due to MM Isoenzyme Only)

Serum Sample	Total MM Activity IU/l	Total MB Activity IU/l	Total Activity of 1st Eluate IU/l	Total Activity of 2nd Eluate IU/l	MB Re- covery %
16	44	736	138	519	70.5
17	22	736	55	533	72.4
18	44	736	110	523	71.1
19	27	491	90	344	70.1
20	39	491	62	356	72.5
21	61	491	96	378	77.0
22	37	245	52	184	75.0
23	7	245	31	164	67.0
24	54	245	74	197	80.4
25	98	98	106	74	75.5
26	58	98	68	76	77.6
27	41	98	53	75	76.5
28	41	49	48	35	71.4
29	44	49	42	37	75.5
30	32	49	28	39	79.6



to the presence of MB isoenzyme in eluate 1 (although not detected by electrophoresis) but may also have been due to activation of creatine kinase by dilution.

When mixtures of the MM and MB isoenzyme of varying composition were analysed, it was found that there was a linear relationship between the amount of MB isoenzyme in the mixture and that determined by the method (Fig. 6-2). As before, however, MB isoenzyme concentration was underestimated by about 25%.

#### Specificity.

The ability of the ion-exchange method to separate the MB from the MM isoenzyme in pathological sera was investigated by analysing eluates 1 and 2 obtained from four sera from patients with acute myocardial infarction. Both eluates (after concentration by pressure dialysis when necessary) after electrophoresis were stained for creatine kinase activity using the agarose film technique. In all cases only the MM isoenzyme was demonstrable in eluate 1 and only the MB isoenzyme in eluate 2.

#### Precision and sensitivity.

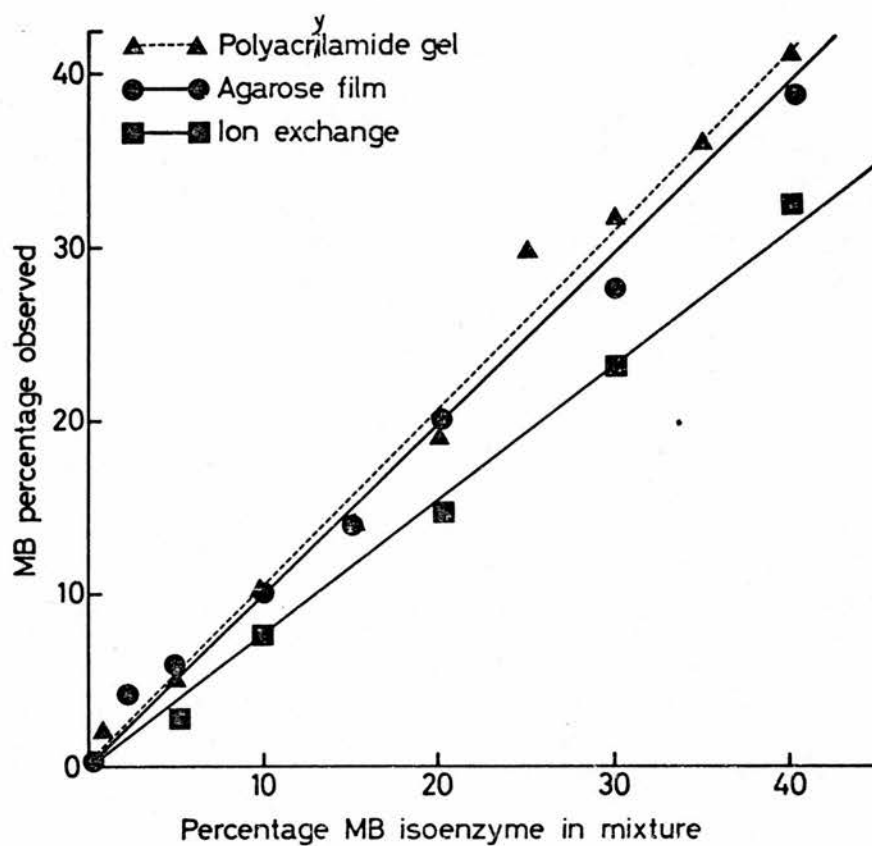
The precision of the ion-exchange method was determined by between-batch repeat analysis of samples from patients, using the formula  $S.D. = \sqrt{\sum d^2 / (2N-1)}$

d = difference between duplicate  
determinations

N = number of pairs of data

Figure 6-2

Analysis of Isoenzyme Composition of Mixtures of Partially  
Purified MM and MB Isoenzymes



The results (Table 6-6) indicate that at low activities the method has relatively poor precision and this limits the useful range within which the method is of value. In practice, activities of less than 10 IU/1 cannot with confidence be distinguished from zero and this figure is, therefore, the practical lower limit of sensitivity of the method. It would be possible with more washing steps and concentration of eluates to increase the sensitivity. At activities over 20 IU/1 the precision was considered satisfactory.

The method is relatively simple to perform and results can be available within about two hours of receipt of the sample.

#### 6-3-2

#### Electrophoretic methods.

Unlike the ion-exchange method these techniques do not yield a direct measurement of MB isoenzyme activity. They measure the proportion of creatine kinase activity present as the MB isoenzyme; their precision and accuracy are, therefore, partly dependent on the measurement of total creatine kinase activity of the specimens.

With both electrophoretic techniques, MB and MM isoenzymes were mixed in varying proportions and the mixtures analysed. The results (Fig. 6-2) indicate that, at least when partially purified enzymes are used, both techniques give a fairly accurate measure of the amount of each isoenzyme present in the mixture.

The precision of the two methods was calculated using between-batch analysis of patients' samples (Table 6-6). The agarose film technique was more precise than the polyacrylamide gel method although both methods, in common with the ion-exchange technique, are imprecise at low MB isoenzyme activities.

The sensitivity of both techniques seemed to be dependent on the extent of serum dilution. For active samples where the serum had to be diluted before application, the sensitivity, when the MB isoenzyme result was reported as a percentage, was higher than for the undiluted sera, presumably due to dilution of interfering substances in the serum.

Sera from patients with myocardial infarction were examined by both electrophoretic methods and by the ion-exchange method whereas the polyacrylamide gel method appeared less satisfactory (Fig. 6-3, Table 6-7).

In terms of technical performance the agarose film method was rapid, taking about  $1\frac{1}{2}$  hours to analyse a batch of 8 samples. The polyacrylamide gel method, on the other hand, was more time consuming, taking about 6 hours for a batch of 18 samples before a result was available. This method also required considerable technical skill if satisfactory results were to be obtained.

6-4

#### Discussion.

The kinetic assay of the MB isoenzyme by differential

Table 6-6

Precision Obtained from Between-batch Analysis of Patients' Specimens  
of the Three MB Isoenzyme Methods

Method	Range (IU/l)		
	0 - 20	20 - 100	100 - 400
Ion Exchange			
Mean	10.4 (5)	55.7 (15)	181 (7)
S.D.	3.5	5.7	7.8
C. V. (%)	33.7	10.2	4.3
Agarose Film			
Mean	8.5 (5)	53.5 (18)	208 (5)
S.D.	1.8	6.2	24.2
C. V. (%)	21.4	11.6	11.6
Polyacrylamide Gel			
Mean	10.6 (5)	55.4 (14)	224 (11)
S.D.	2.8	9.7	33.7
C. V. (%)	26.1	17.5	15.0

In parentheses is the number of samples analysed.

Figure 6-3

Correlation between the Agarose Film and Ion-exchange Methods  
of Determining Serum MB Isoenzyme Activity

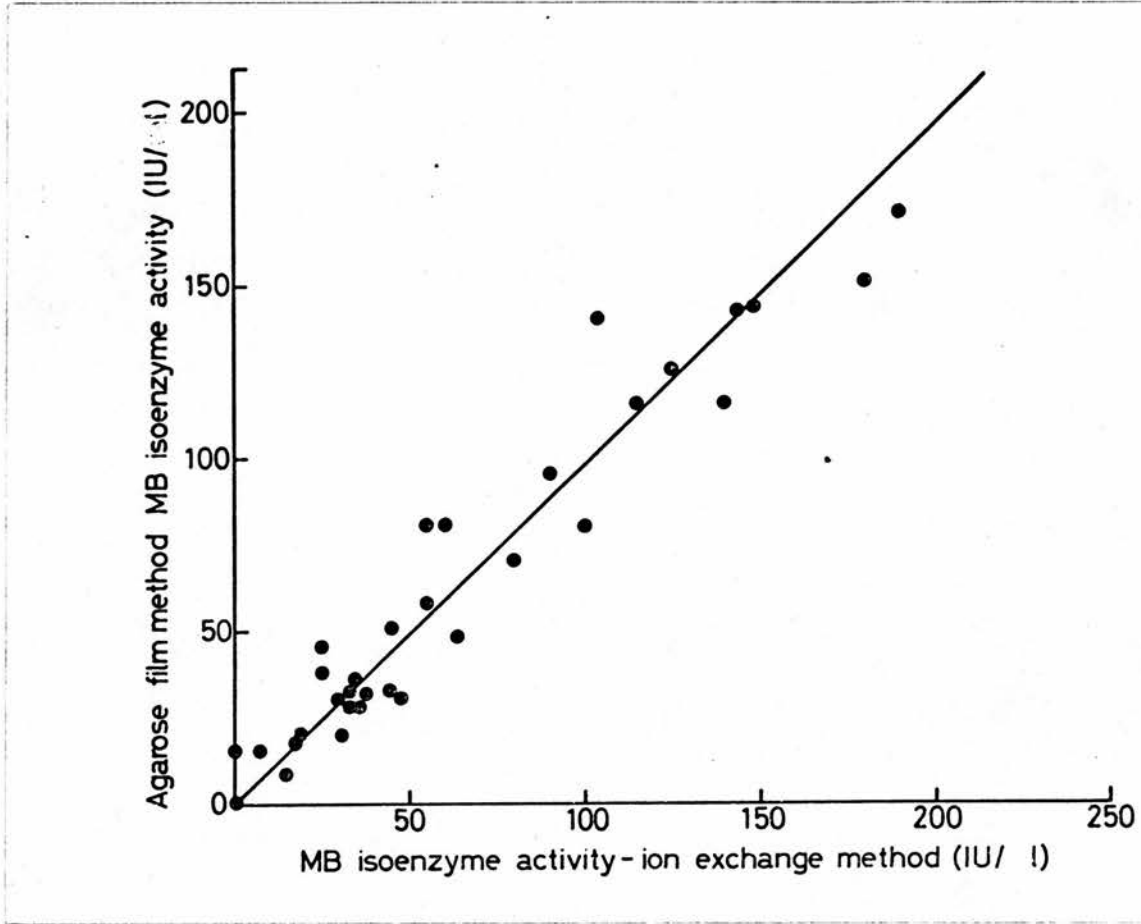


Table 6-7

Correlation of Electrophoretic Methods with DEAE Sephadex  
Ion-exchange Method

	No. of Data Pairs	Equation Parameters ( $y = ax + b$ )		Correlation Coefficient (r)
		a ( $\pm$ S.D.)	b ( $\pm$ S.D.)	
Agarose Film Method	36	1.01 (0.03)	-1.4 (3.5)	0.98
Acrylamide Gel Method	23	0.96 (0.1)	+5.9 (0.59)	0.90

inactivation by pHMB seems to work well in purified extracts but is not applicable to serum, presumably due to the high concentration of protein and other substances present in serum. Further dilution of serum in the incubation mixture is not practicable as the amount of MB isoenzyme present, already small, might be diluted below the limit of sensitivity of the method. This, together with the rapid reactivation of both isoenzymes in the assay mixture has made it impracticable to develop a method based on this differential inactivation.

Methods based on differences in substrate affinity alone are often unsuitable for routine use since large differences in substrate affinity are required; such differences may be affected by the presence of various serum constituents. Moreover, substrate affinities of enzymes present in organ extracts and serum are not always identical. In some cases, e.g. malate dehydrogenase, the substrate affinity is not even a constant value and it may fall after the release of the enzyme into plasma (Schmidt and Schmidt, 1960). Nevertheless, one such method had been reported for the MB isoenzyme (Witteveen et al., 1974).

It seems likely that many Clinical Chemistry laboratories may be requested to provide measurements of the activity of the MB isoenzyme of creatine kinase, since in certain circumstances, this enzyme test has unique advantages over other currently available "cardiac enzymes" (Section 4). Unfortunately, at present, no one method of measurement appears altogether satisfactory. Any test considered for



routine use should be accurate and precise but should also be cheap and relatively simple to perform.

Most existing methods for measuring the MB isoenzyme are insufficiently sensitive to detect the amounts normally present in human serum, although it has recently been reported that a chromatographic technique using DEAE cellulose is suitable for this purpose provided the serum samples are concentrated prior to analysis (Nealon and Henderson, 1975). None of the methods described in this thesis is sensitive enough to detect MB isoenzyme levels of 0-6 IU/l, the reference values suggested by Nealon and Henderson. It is not at present apparent how important this lack of sensitivity may be, since the main advantage of MB isoenzyme measurement seems to be its specificity for cardiac tissue rather than its ability to detect minor degrees of myocardial damage. MB isoenzyme measurements offer a rapid, effective method of distinguishing patients with serum enzyme elevation due to myocardial infarction from those with enzyme elevation due to other causes. For these purposes, a semi-quantitative result may be all that is required.

The polyacrylamide gel technique has been used as a semi-quantitative method for some years, mainly for research purposes. However, it is rather tedious and the present quantitative method developed from it is significantly less precise than the other methods we have tested. Most of the difficulties relate either to the difficulty of introducing the coupling enzymes, glucose-6-phosphate

dehydrogenase and hexokinase, into the separating gel in a sufficiently uniform manner, or to non-specific reduction of the phenazine methosulphate/nitroblue tetrazolium mixture by the thiol agents included in the solution in order to activate creatine kinase. The major advantage of the polyacrylamide gel technique is that high resolution of the isoenzyme bands is readily obtainable, but it seems unlikely that this could be a useful feature in routine diagnostic work.

The commercial agarose film method, on the other hand, has proved simple, reliable and rapid. Although the method is not claimed to be quantitative it has been possible to obtain reasonable quantitation by scanning the fluorescent bands obtained.

The results with partially purified isoenzymes suggest that the ion-exchange method should give low results for serum MB isoenzyme activity, since only 75% of added MB isoenzyme was "recovered" from serum. In fact, the ion-exchange method correlated well with both electrophoretic methods, yielding results of the same magnitude. It seems unlikely that the electrophoretic methods underestimate MB isoenzyme activity since accurate results were obtained when mixtures of purified isoenzymes were analysed. It is more probable that the MB isoenzyme preparations added to the serum did not behave in the same manner as endogenous creatine kinase isoenzymes. However, it is difficult to be certain about the accuracy of all these procedures since loss or denaturation of one or both isoenzymes during

separation may be completely or partially compensated by the effects of serum dilution on creatine kinase activity.

Apart from problems of lack of sensitivity all three methods are able to detect and quantitate the MB isoenzyme of creatine kinase. Experience with MB isoenzyme determination in a fairly large series of patients with myocardial infarction suggests that all such patients will attain serum activities of the MB isoenzyme readily quantifiable by these techniques.

The practical problems of selecting a suitable technique for routine use will depend on a number of factors, such as cost, convenience, analytical performance and the patient population to be studied. The presently available evidence suggests that column chromatography on DEAE cellulose or Sephadex may be the most accurate method of determination. However, even if the simplified techniques described (Mercer, 1974; Nealon and Henderson, 1975) are used, the method may be rather time consuming and require creatine kinase analysis on several eluate tubes. Of the methods compared in the present study, the polyacrylamide gel technique is less accurate, less precise and requires greater technical skill than the other two methods. The agarose film technique (and possibly other similar commercial techniques) is fairly rapid and appears reasonably precise and accurate. The batch method using DEAE Sephadex, can be performed in about the same time, is cheaper and requires a minimum of special apparatus, but may need 1 ml of serum. It is felt that either of these methods would be suitable for use in a routine

laboratory and would provide clinical information not obtainable at present using other enzyme tests.

SECTION 7

CONCLUSION

The study of the tissue specificity of isoenzymes has found many useful applications in clinical enzymology since tissue specific isoenzymes often indicate the site of a disease process. The use of different isoenzymes of lactate dehydrogenase in the diagnosis of diseases affecting different tissues illustrates the success of such studies. However, it is extremely rare to find an isoenzyme which is completely specific to one tissue: it is necessary for the clinical enzymologist to investigate the distribution of isoenzymes in different tissues and their release in various disease states. In this way the practical advantages to be gained from isoenzyme studies on serum may be assessed. It is with this aspect of tissue specificity that the present studies on the MB isoenzyme of creatine kinase have been concerned.

Most of the clinical studies on the tissue specificity of isoenzymes have been concerned with enzymes which are present in the cytoplasm of the cell. However, it is known that mitochondrial and membrane-bound enzymes may be released under certain pathological circumstances (Schmidt et al., 1967a; Boyde, 1968a). Much of the evidence suggests that mitochondrial isoenzymes are released only when cellular damage is more severe than that necessary to cause the release of soluble cytoplasmic enzymes. Thus another aspect of the present study has been to measure the release of mitochondrial isoenzymes into the blood of patients with myocardial infarction.

The investigations into the release of the MB isoenzyme

have shown that measurement of the activity of this enzyme in serum provides a more specific and sensitive test for myocardial infarction than any of the other enzymes studied (mAST, cAST, mMD, cMD, CK-MM, USLD and GLDH). Furthermore, it was the only enzyme that could reliably help to diagnose myocardial infarction after surgical operation. The activity of the MB isoenzyme in serum reached a peak value earlier and returned to normal earlier than any of the other enzymes studied. Consequently MB isoenzyme measurement is particularly valuable in detecting the occurrence of reinfarction. On the other hand, the test is of less value than other enzyme tests in patients in whom it is not possible to obtain blood specimens within 36 hours of suspected infarction.

The results of the clinical study on patients with suspected myocardial infarction indicate that the mitochondrial isoenzymes of aspartate aminotransferase (mAST) and malate dehydrogenase (mMD) were of less value in establishing a diagnosis of infarction than the corresponding cytoplasmic isoenzymes (cAST and cMD). The serum activity of the mitochondrial isoenzymes rose more slowly and reached a peak later than the cytoplasmic isoenzymes. Furthermore, the peak activity did not correlate well with the corresponding peak activity of the cytoplasmic isoenzymes. It is possible that this poor correlation was due to release of cytoplasmic, but not mitochondrial, isoenzymes from cells which suffered reversible damage in the early stages of the disorder. However, the peak value of mitochondrial isoenzyme activity

was found to correlate better with post-infarct heart failure than the cytoplasmic isoenzymes. Therefore, it is possible that the mitochondrial isoenzymes may be better indices of the severity of the infarction than the cytoplasmic isoenzymes. In the absence of reliable data about blood half-life of all the enzymes being studied, it was not possible to determine definitively whether the release of mitochondrial enzymes continued after the release of cytoplasmic enzymes had ceased.

The biochemical studies on the partially purified isoenzymes from human heart tissue show that the MB isoenzyme is less stable to heat, to denaturation by urea and to extreme pH values than the MM isoenzyme. The MB isoenzyme had a lower  $K_m$  value for both creatine phosphate and MgADP than the MM isoenzyme. Also the  $K_m$  values of the two isoenzymes behaved differently when the pH of the reaction mixture was varied. Apart from these quantitative differences, in many other respects the two isoenzymes showed similar properties, for example in the type of reaction mechanism, behaviour with many inhibitors and with thiol compounds. In most respects, as stated above, the MM isoenzyme was more stable than the MB isoenzyme, but, under suitable experimental conditions, the MM isoenzyme was almost completely inhibited by p-hydroxymercuribenzoate while about 50% of the activity of the MB isoenzyme remained. It was impossible to reproduce this differential inhibition of the MM isoenzyme when the isoenzymes were present in serum rather than in purified extracts, so the method could



not be used as the basis for a test of clinical value.

From the study of the more conventional separative methods, a batch ion-exchange method was developed and found to compare favourably with the polyacrylamide gel method used in this study and with a commercial electrophoretic method using agrose film. In certain circumstances, depending on the equipment, staff and finance available, the batch ion-exchange method could prove to be the method of choice for the routine measurement of the activity in serum of the MB isoenzyme of creatine kinase.

Although the MB isoenzyme has proved the most sensitive and specific test at present available for the diagnosis of myocardial infarction, it is not certain how long this will remain true. Clinical enzymology, in common with other disciplines, continues to develop new methods which add to the value of those which have previously been found useful. In this way, the original descriptions of the value of AST and LD in the diagnosis of myocardial infarction (LaDue et al., 1954; Wroblewski and LaDue, 1955) were later supplemented by the use of LD isoenzyme studies (Wroblewski et al., 1960) and the introduction of serum creatine kinase measurements (Dreyfus et al., 1960). Since then, few new enzymes of diagnostic value for myocardial infarction have been discovered. Instead much effort has been spent on improving the precision, sensitivity and tissue specificity of the existing enzyme assays.

There has been a less obvious but equally important process of consolidation during the past ten years -

techniques of enzyme assay have been improved, reference ranges have been measured more accurately, and possible pitfalls in interpretation have been clarified. The process is not yet complete, even for aspartate aminotransferase, since clinical enzymologists are only now reaching agreement on the experimental conditions suitable for a reference method. It is apparent that much more information about the use of the MB isoenzyme will be required, both in terms of methodology and in terms of evaluation of its place in clinical diagnosis.

One area currently of great clinical interest is the use of serum enzymes to estimate infarct size (Shell et al., 1973). These workers have used serial serum enzyme measurements obtained in the first few hours after infarction to predict the rest of the serum enzyme time curve. In the absence of therapeutic intervention, these workers found that the "predicted" and "observed" curves were similar, and were therefore able to predict likely infarct size within about 12 hours of infarction. Shell et al. (1973) also found that they were able to reduce the "observed" infarct size to less than the "predicted" value by appropriate measures such as hypotensive therapy. The enzyme selected for such evaluations should be specific for the myocardium, but the other necessary characteristics are less easy to assess and may depend on the purpose for which the evaluation of infarct size is required.

If a retrospective assessment of infarct size is to be carried out for prognostic or other reasons, it would seem

desirable that an enzyme liberated only from irreversibly damaged or dead cells should be used. Possibly one of the mitochondrial enzymes would be most suitable for this purpose, at least on theoretical grounds. If, on the other hand, serum enzyme activities are to be used to assess the effectiveness of therapeutic measures designed to reduce infarct size (Shell and Sobel, 1974), then an enzyme which is released into the plasma early in the disease, and which is released from reversibly as well as irreversibly damaged cells, is required. The MB isoenzyme of creatine kinase seems to fulfil this second set of requirements fairly well. In practice it seems likely that there will be many difficulties, partly because of biological variation in the rate of release and inactivation of enzymes, and partly due to difficulties in obtaining sufficiently precise and accurate measurements of enzyme activity.

Present knowledge about the biochemical factors contributing to the release of enzymes from damaged myocardium is very limited. Since many of the enzyme tests have been established on an empirical basis, it is possible that unexpected problems might arise with further improvements in the sensitivity and specificity of methods of enzyme assay. It has been pointed out that improvements in methods of assay could, in some cases, remove chemical "artefacts" in the assay system that contribute to the clinical significance of the result. For example, renin when assayed after separation from serum correlates less well with the presence of hypertension than when assayed in untreated

serum, although the latter method of assay is less precise and accurate (Goodfriend, 1970). It is possible that in some diseases it is of more diagnostic value to measure enzyme activity than enzyme concentration because of the presence of at present unknown activating or inhibiting factors in serum. A more accurate method of measurement of enzyme concentration might no longer be affected by such a factor and hence the test would be of less diagnostic value.

It is not certain whether these reservations are relevant to the present studies on enzyme release in myocardial infarction. However, it seems unlikely that the maximum diagnostic value will be obtained until more is understood about those factors which control the release of enzymes from damaged cells and about other metabolic and adaptive changes which may occur in such cells. Some studies of this nature have already been carried out. Niles and Barnhouse (1967) studied myocardial cells in heart tissue slices and observed that prolonged anoxia caused disruption of the bonds between lipid and protein. Gudbjarnasson and co-workers (1967) studied the activity of myocardial enzymes from various sites in the cell after myocardial infarction. They found a sharp decrease in the activity of glycolytic and oxidative enzymes in mitochondria but a large increase in the activity of the hexose monophosphate shunt enzymes. More recently, it has been suggested that the ATP content of the cell and the presence of phospholipases in the surrounding inflammatory fluid were

important factors controlling the release of enzymes from injured cells (Wilkinson and Robinson, 1973; Robinson and Wilkinson, 1974).

It is clear that the metabolic events which occur close to and within an area of ischaemic or infarcted myocardium are of great importance to doctors wishing to improve the prevention, diagnosis and management of the condition. The release of enzymes is only one biochemical event among many, but it seems likely that there is a close inter-relationship between all the factors involved. In addition to their purely diagnostic role, studies on enzyme release may play their part in unravelling other problems associated with the metabolism of ischaemic tissue. For this purpose it might be profitable for future work to study the mitochondrial isoenzyme of creatine kinase as it is closely linked with the energy metabolism of the cell. These studies would include the investigation of its biochemical properties, its functions in mitochondria during normal and anoxic conditions, and its pattern of release from the myocardium after infarction.

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APPENDIX 1

Abbreviations

The following abbreviations have been used in the text:-

a. Enzymes

<u>Trivial name</u>	<u>Systematic name</u>	<u>Code number</u>
Acid phosphatase	Orthophosphoric monoester hydrolase	3.1.3.2
Adenylate kinase	ATP:AMP phosphotransferase	2.7.4.3
Alanine aminotransferase (ALT)	L-alanine:2 oxoglutarate amino-transferase	2.6.1.2
Aldolase	Ketose-1-phosphate aldehyde lyase	4.1.2.7
Alkaline phosphatase	Orthophosphoric monoester hydrolase	3.1.3.1
Aspartate aminotransferase (AST)	L-aspartate: 2 oxoglutarate aminotransferase	2.6.1.1
ATPase	ATP phosphohydrolase	3.6.1.4
Creatine kinase (CK)	ATP:creatine phosphotransferase	2.7.3.2
Glucose-6-phosphate dehydrogenase (G6PD)	D-glucose-6-phosphate: NADP oxidoreductase	1.1.1.49
Glutamate dehydrogenase (GLDH)	L-glutamate:NAD oxidoreductase	1.4.1.2
Glyceraldehyde 3-phosphate dehydrogenase	D-glyceraldehyde-3-phosphate: NAD Oxidoreductase	1.2.1.12
Hexokinase (HK)	ATP: D-hexose-6-phosphotransferase	2.7.1.1
Isocitrate dehydrogenase	L-isocitrate: NADP oxidoreductase (decarboxylating)	1.1.1.42
Lactate dehydrogenase (LD)	L-lactate:NAD oxidoreductase	1.1.1.28
Malate dehydrogenase (MD)	L-malate:NAD oxidoreductase	1.1.1.37
Pyruvate kinase (PK)	ATP: pyruvate phosphotransferase	2.7.1.40

b. Chemicals

<u>Abbreviation</u>	<u>Full name</u>
ADP	Adenosine-5-diphosphate
AMP	Adenosine-5-monophosphate
ATP	Adenosine-5-triphosphate
DTT	Dithiothreitol
EDTA	Ethylenediaminetetra-acetic acid
GSH	Reduced glutathione
GSSG	Oxidised glutathione
NAD	Nicotinamide - adenine-dinucleotide
NADH	Nicotinamide-adenine-dinucleotide (reduced form)
NADP	Nicotinamide-adenine-dinucleotide phosphate
NADPH	Nicotinamide-adenine-dinucleotide phosphate (reduced form)
NBT	Nitroblue tetrazolium
pHMB	para-hydroxy-mercuribenzoate
PMS	Phenazine methosulphate
Tris	tris-(hydroxymethyl) amino-methane



## APPENDIX 2

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### APPENDIX 3

#### Publications

The following work described in this thesis has been published:

- \*1. Smith A.F., Radford D., & Wong P.C-P. (1975) "Creatine kinase isoenzyme studies in myocardial infarction." Clin. Chem. 21, 975
  2. Wong P.C-P. & Smith A.F. (1975) "Comparison of 3 methods of analysis of the MB isoenzyme of creatine kinase in serum." Clin. Chim. Acta 65, 99-107
  3. Wong P.C-P. & Smith A.F. (1976) "Biochemical differences between the MB and MM isoenzymes of creatine kinase." Clin. Chim. Acta 68, 147-158
  4. Smith A.F., Radford D., Wong P.C-P. & Oliver M.F. (1976) "Creatine kinase isoenzyme studies in the diagnosis of myocardial infarction." British Heart J. 38, 225-232
  5. "The release of some mitochondrial enzymes in myocardial infarction" in preparation.
  6. "An assay method for malate dehydrogenase using malate as substrate." in preparation.
- \* Abstract of a paper presented at the 9th International Congress of Clinical Chemistry.